ELECTROPHORETIC AND IMMUNOCHEMICAL IDENTIFICATION OF SEAFOOD SPECIES USING SHRIMP AS A MODEL

BY

HAEJUNG AN

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL OF
THE UNIVERSITY OF FLORIDA
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1989

To my parents,

Mr. Sungho Ahn and Ms. Seung-ah Park

With Love and Gratitude

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to Dr. Cheng-i Wei, the chairman of my graduate committee, for his excellent leadership, support, and encouragement without whom the accomplishment of this project was impossible. I would like to extend my gratitude to Dr. Paul A. Klein for his special assistance and suggestions to improve the depth of this project. My special thanks are extended to Drs. Maurice R. Marshall, W. Steven Otwell, and Kuo-Jang Kao, for their assistance, advice and support.

Finally, my special thanks are extended to my husband, Mr. Thomas A. Seymour, for his patience, love, and willingness to share the joy and sorrow; and to my parents, Mr. and Mrs. Sungho Ahn, who are always with me in heart, for their deep faith and love.

TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS iii

LIST OF TABLES vii

LIST OF FIGURES viii

ABSTRACT xii

CHAPTERS

I. INTRODUCTION 1

II. REVIEW OF THE LITERATURE 6

Electrophoretic Identification Methods 6

Principles of Electrophoretic Methods ... 6

Polyacrylamide gel electrophoresis 6
Sodium dodecyl sulfate-polyacrylamide gel electrophoresis 8
Isoelectric focusing 9
Two-dimensional gel electrophoresis 10

Application of Electrophoretic Methods for Species Identification 11

Immunological Identification Methods 14

Principles of Immunological Assays 15

Precipitin reaction 15
Ouchterlony double diffusion 15
Agglutination 16
Immunoelectrophoresis 16
Indirect assays 16
Western blotting 17

Principles of Hybridoma Technique 18

Application of Monoclonal Antibodies in Immunochemical Identification Techniques 21

Chromatographic Identification Methods 23

Principles of Chromatographic Methods ... 23

Liquid-liquid chromatography 24
Liquid-solid chromatography 25
Ion-exchange chromatography 25
Ion-pair chromatography 26
Exclusion chromatography 26
Affinity chromatography 27

Application of Chromatographic Methods in Species Identification 27

Shrimp Species Identification by Conventional Method 28

III ELECTROPHORETIC IDENTIFICATION OF RAW AND COOKED SHRIMP USING VARIOUS PROTEIN EXTRACTION SYSTEMS

Introduction 33
Materials and Methods 35
Results and Discussion 39
Conclusion 57

IV SPECIES IDENTIFICATION OF RAW AND BOILED SHRIMP BY A UREA GEL ISOELECTRIC FOCUSING TECHNIQUE

Introduction 59
Materials and Methods 61
Results and Discussion 65
Conclusion 76

V USE OF A MODIFIED UREA GEL ISOELECTRIC FOCUSING TECHNIQUE FOR SPECIES IDENTIFICATION OF RAW AND BOILED SHRIMP

Introduction 78
Materials and Methods 79
Results and Discussion 83
Conclusion 92

VI IMMUNOCHEMICAL IDENTIFICATION OF SHRIMP SPECIES USING ROCK SHRIMP AS A MODEL

Introduction 95
Materials and Methods 97
Results and Discussion 106
Conclusion 140

VII SUMMARY AND RECOMMENDATIONS 141

APPENDIX

ELECTROPHORETIC IDENTIFICATION OF FISH SPECIES USED IN SURIMI PRODUCTS

Introduction 145
Materials and Methods 147
Results and Discussion 151
Conclusion 164

REFERENCES 165

BIOGRAPHICAL SKETCH 177

LIST OF TABLES

Table Page

1. Protein of the various shrimp extracts 42
2. Protein recovery of water, 1% SDS and 8 M urea in extracting shrimp protein 44
3. The efficiency of the SDS-PAGE assay in determining the percent content of rock shrimp in various mixtures by a blind study 53
4. Composition of various unknown mixture samples used in Blind Study II 107
5. The relative mobility and percent area of the various rock shrimp protein bands on the SDS-PAGE gel as determined by a gel scanner 113
6. Screening of hybridoma master cultures by ELISA for reactivity with protein "C" and various shrimp extracts 116
7. Screening of cloned hybridoma cultures by ELISA for reactivity with protein "C" and various shrimp extracts 121
8. The reactivity of 4H2-10D3 McAb with the water extracts of the various seafood and meat samples in a blind study using the ELISA test 137
9. Sensitivity limit of the ELISA test in determining rock shrimp content in mixture samples 139

LIST OF FIGURES

Figure Page

1. Metabolic pathways relevant to hybrid selection in medium containing hypoxanthine, aminopterin and thymidine (HAT) medium 20
2. Schematic drawing of shrimp in lateral view 29
3. SDS-PAGE patterns of raw white shrimp extracted with water, water homogenates adjusted to pH 8.0, 0.1 M NaCl, 1% SDS, and 8 M urea 40
4. SDS-PAGE patterns of cooked white shrimp extracted with water, water homogenates adjusted to pH 8.0, 0.1 M NaCl, 1% SDS, and 8 M urea 41
5. SDS-PAGE patterns of water and SDS extracts of raw pink, white and rock shrimps and their mixtures 46
6. SDS-PAGE patterns of SDS extracts of cooked pink, white and rock shrimp and their mixtures 50
7. SDS-PAGE patterns of pink shrimp extracted in the presence and absence of protease inhibitor, PMSF 54
8. Serial changes in SDS-PAGE patterns of raw pink and rock shrimp extracted with 8 M urea 56
9. SDS-PAGE patterns of urea extracts of raw rock shrimp with or without dilution 58
10. Protein banding patterns of the water extract of raw pink shrimp and protein standards focused on polyacrylamide gel containing 0, 6, or 9.2 M urea 66

11. IEF patterns of the water extract of raw pink shrimp and protein standards focused on polyacrylamide gel containing 5% ampholyte for 17 hr, 6.2% ampholyte for 10 hr, and 6.2% ampholyte for 17 hr 67

12. IEF patterns of raw and cooked pink shrimp extracted with water, 1% SDS, and 8 M urea 69

13. IEF patterns of water extracts of raw pink, white, and rock shrimp, and their mixtures 72

14. IEF patterns of SDS extracts of cooked pink, white, and rock shrimp, and their mixtures 73

15. IEF patterns of water extracts of cooked pink, white, and rock shrimp, and their mixtures 74

16. pH profiles of IEF gel containing three different ratios of wide and narrow pH range of ampholytes 85

17. IEF patterns of water extracts of raw pink and protein standards focused on urea polyacrylamide gel containing 100%, 80%, and 60% of pH 4-6.5 ampholytes with addition of 0%, 20%, and 40% pH 3-10 87

18. IEF patterns of water extracts of raw pink, white, and rock shrimp, and their mixtures 88

19. Densitometric profiles of water extracts of pink, white, and rock shrimp extracted with water 90

20. IEF patterns of water extracts of cooked pink, white, and rock shrimp, and their mixtures 91

21. IEF patterns of SDS extracts of cooked pink, white, and rock shrimp, and their mixtures 93

22. SDS-PAGE profiles of water-soluble proteins from various seafood and meat 108

23. SDS-PAGE profiles of water-soluble proteins from various shrimp and lobsters 110

24. Two dimensional gel electrophoresis pattern of rock shrimp extracted with water 111

25. Densitometric scanning profile of the water-soluble rock shrimp proteins analyzed on a SDS-PAGE gel 112

26. SDS-PAGE patterns of the eluted rock shrimp protein "C" and the MW protein standards 115

27. Screening of the uncloned hybridoma cultures by immunodot blotting for reactivity with rock shrimp extracts added with SDS 117

28. Screening of monoclones derived from the 4H2 master culture by immunodot blotting for reactivity with shrimp extracts containing 0.1% SDS 120

29. Determination of the specificity of 4H2-10D3 McAb in reacting with rock, pink and white shrimp as well as MW protein standards as analyzed by the Western blotting technique 126

30. Determination of the specific reactivity of 4H2-10D3 McAb with rock shrimp as analyzed on immunodot blot with the water extracts of various shrimp and lobsters as well as various seafood and meat 127

31. Effect of the heat treatment, the addition of SDS or the combination of both on the reactivity of rock shrimp extract with 4H2-10D3 McAb as analyzed by immunodot blot 130

32. Effect of heat treatment, the addition of SDS or the combination of both on the increment of reactivity of rock shrimp proteins with 4H2-10D3 McAb on ELISA 131

33. Dose-related changes of the reactivity of 4H2-10D3 McAb with rock shrimp extract at 5 μg/mL by ELISA 134

34. Dose-related changes of the reactivity of rock shrimp extract with the 4H2-10D3 McAb at 50 and 100 μg/mL by ELISA 135

35. SDS-PAGE patterns of intact fillet and surimi of Alaska pollock and red hake extracted with water 152

36. IEF patterns of intact fillet and surimi of Alaska pollock and red hake extracted with water 153

37. SDS-PAGE patterns of cooked fillet and/or surimi of Alaska pollock and red hake extracted with water 156

38. IEF patterns of cooked fillet and/or surimi of Alaska pollock and red hake extracted with water 157

39. SDS-PAGE patterns of cooked fillet and/or surimi of Alaska pollock and red hake extracted with SDS 159

40. SDS-PAGE patterns of cooked fillet and/or surimi of Alaska pollock and red hake extracted with urea 160

41. IEF patterns of cooked fillet and/or surimi of Alaska pollock and red hake extracted with SDS 162

42. IEF patterns of cooked fillet and/or surimi of Alaska pollock and red hake extracted with urea 163

Abstract of Dissertation Presented to the Graduate School of
the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

ELECTROPHORETIC AND IMMUNOCHEMICAL IDENTIFICATION OF SEAFOOD SPECIES USING SHRIMP AS A MODEL

By

Haejung An

May, 1989

Chairman: Cheng-i Wei
Cochairman: Maurice R. Marshall
Major Department: Food Science and Human Nutrition

Identification of shrimp species was achieved by comparing the electrophoretic protein patterns using either sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or isoelectric focusing (IEF), or by detecting components with species-specific monoclonal antibodies (McAb) using an enzyme-linked immunosorbent assay (ELISA) or immunodot blot. An appropriate extractant assisted in identification of shrimp species particularly for cooked samples by solubilizing heat-denatured proteins. Water extraction showed banding patterns highly species-specific for raw shrimp, while SDS extracts provided the greatest information on genus variation of cooked samples.

The incorporation of 9.2 M urea in IEF polyacrylamide gels containing pH 3-10 ampholytes greatly increased protein resolution after 17 hr focusing time. Since the important proteins for shrimp species identification were resolved within a narrow pH range of 5.11 - 6.75, the incorporation of an ampholyte mixture (20% pH 3-10 and 80% pH 4-6.5) in the gel provided the best separation of shrimp proteins. Identification of shrimp species was achieved using these conditions in both pure and mixture samples through detection of species-specific protein bands. Water extracts of raw shrimp and SDS extracts of cooked shrimp showed excellent banding patterns useful for species differentiation. However, the detection of cooked shrimp in a mixture was difficult except for rock shrimp.

A protein designated as "C" was found from the SDS-PAGE protein banding patterns to be specific for rock shrimp when compared with those of seafood or meat samples. This protein was isolated from the 12% polyacrylamide gels and used to develop McAb against rock shrimp. The highly rock shrimp-specific McAb (4H2-10D3) recognized the antigenic determinant(s) present on protein "C" in both the native and heat-denatured rock shrimp extract. Specificity of the McAb was confirmed by testing against pink, white and rock shrimp protein extracts using the Western blotting technique and against 23 types of commonly consumed seafood and meat using ELISA and immunodot blotting. ELISA assay using the 4H2-10D3 McAb was proven to be effective and sensitive in a blind study to identify rock shrimp and to detect its presence in mixtures.

CHAPTER I
INTRODUCTION

There has been a tremendous growth in seafood consumption due to changes in consumer attitudes toward health and nutrition. Compared to red meat, seafood has been well recognized for its low fat and cholesterol content as well as high amount of n-3 polyunsaturated fatty acid (Kinsella, 1988). As a result, seafood has been recommended in diets as a meat substitute. Domestic seafood landings have been limited, and seafood supply relies heavily on imports from other countries (Vondruska, 1988). The U.S. seafood market therefore abounds with numerous types and species of finfish and shellfish.

Species identification can be difficult. People from different geographical regions use different nomenclature for the same species of finfish or shellfish. For example, one type of perch (Perca flavescens) is referred to as perch, yellow perch, river perch, American perch, ringed perch, and striped perch depending on the locations (Martin et al., 1983). This species identification problem is further complicated because people also use the same common name for different species; drum (Bairdiella chrysura) and sea bass (Diplectrum formosum) are sometimes referred to as silver perch (Martin et al., 1983).

Finfish and shellfish can easily lose their identity after filleting, mincing, or cooking. It is therefore easy to substitute lower quality species for the expensive or limited species. Unintentional fraud can occur when species identification is done by untrained personnel, and the resulting products are declared misbranded and/or adulterated by regulatory agencies (Vondruska, 1988).

Quantitative identification of seafood species in fabricated or processed seafood products also deserves attention. Surimi-based seafood products such as crab meat, shrimp, and lobster analogs have been recently introduced into the seafood market; some of these products are claimed to contain up to 35% natural seafood (Regenstein and Lanier, 1986). However, there are no analytical methods available for quantitative identification of the natural species to test the claims or to assure the quality of the processed seafood products.

Several official electrophoretic methods, such as starch gel-zone electrophoresis, acrylamide disc electrophoresis, thin layer polyacrylamide gel isoelectric focusing and cellulose acetate strip, have been approved for use by the Association of Official Analytical Chemists (AOAC, 1984) to differentiate species of seafood or seafood products. By comparing the electrophoretic protein profiles of the fish species to the data bank standards, various species have been identified. However, the data bank collection of the authentic fish species should be as complete as possible in order to provide the highest efficiency for species identification.

Electrophoretic methods have some limitations. These methods are laborious, time-consuming and require a substantial amount of equipment; they are therefore limited for application in field testing. The existence of polymorphism within one fish species also complicates the identification process. For example, 16 distinct variations in protein patterns were observed for monkfish based on 4 protein bands; therefore, it is necessary to compare the unknown pattern with the possible variant patterns to effect a reliable identification (Lundstrom, 1981a).

Electrophoretic methods are limited in identifying the species origin of cooked or processed seafood. The available methods are designed mainly for raw fish and are not suitable for cooked or processed seafood. Protein molecules denature irreversibly and aggregate after heat treatment (Foegeding, 1988). Therefore, there has been a growing need to develop fast, reliable and accurate methods to identify and quantitate species of intact as well as processed seafood for regulatory purposes in order to protect consumers' value.

Immunological assays can replace electrophoretic methods to reduce analysis time and cost, as well as to increase the sensitivity in detecting food components. The immunological techniques, such as immunodiffusion, hemagglutination inhibition, rocket immunoelectrophoresis, and enzyme-linked immunosorbent assay (ELISA) have been widely used for detecting species origin of fish, beef, pork, and chicken or to detect food components in seafood or meat products (Hayden, 1977, 1978, and 1981; Hitchcock et al., 1981; Karpas et al., 1970; Skerritt, 1985). ELISA offers advantages in that (1) the test can be performed in a short time, (2) a small volume of species-specific antisera is required, (3) the antisera can be mixed for simple screening procedure, (4) equipment is available to semi-automate the assay, and (5) the increased sensitivity of the assay allows the use of simple sampling techniques (Whittaker et al., 1983). However, the ELISA technique has not been routinely applied to differentiate species in meat samples because of the cross-reactivity of conventional antisera with unrelated species and the inconsistent specificity of the antisera from one batch to another (Kang'ethe et al., 1982; Patterson et al., 1984; Whittaker et al., 1983). These problems with conventional antisera can be alleviated by the use of monoclonal antibodies specific for different food components. The hybridoma technique first introduced by Kohler and Milstein (1975) can be used to produce large quantities of monoclonal antibodies (McAb) with exceptional specificity and consistent binding properties (Edwards, 1981). Therefore McAbs can be used effectively in ELISA tests to identify species origin of the components as well as to quantitate their levels in meat or seafood products.

The objectives of this study are as follows:

(1) To determine if the use of an effective protein extractant system can improve protein extractability from raw and cooked shrimp, and enhance protein band resolution in the electrophoretic gels.

(2) To improve the existing isoelectric focusing (IEF) methods by incorporating urea and ampholyte mixtures in the gel so that better protein resolution can be achieved and used for seafood species identification.

(3) To produce species-specific McAbs against rock shrimp proteins isolated by using the methods learned from objectives (1) and (2) (these McAbs are expected to show high specificity for antigenic determinants and reduced cross-reactivity).

(4) To optimize the immunochemical assay conditions for identifying seafood species and quantitation (immunodot blot and ELISA test conditions are used and optimized to enhance the sensitivity and accuracy).

The contents presented in chapters 3-6 and appendix are each a complete study and can be published as an individual paper with specific objectives, methods of approaches and defined results and discussion.

CHAPTER II
REVIEW OF THE LITERATURE

The following discussion will review the currently available methods for protein separation and/or detection, which can be applied for identification of seafood species. The principles of protein separation and/or detection will be discussed for each method, followed by applications for species identification. A brief description of the conventional shrimp identification method is also included for comparison.

Electrophoretic Identification Methods

Proteins can be effectively separated by electrophoresis because they differ in molecular size, charge and isoelectric points. Electrophoretic methods are the only official method currently approved for fish species identification (AOAC, 1984). By comparing the protein profiles of meat or seafood samples with those of authentic samples, species origin of the food can be determined.

Principles of Electrophoretic Methods

Polyacrylamide gel electrophoresis. Proteins possess a charge as a result of their component amino acids. When a charged molecule is placed in an electric field, the molecule is attracted to and migrates toward the electrode. The velocity of the molecular movement is proportional to the field strength and charge on the molecule but inversely proportional to its size and viscosity of the solution (Cooper, 1977).

The behavior of a protein subjected to electrophoresis is governed mainly by its charge at a particular pH. At the isoelectric point (pI), positive and negative charges are equal, and the protein exhibits zero net charge; therefore, no migration takes place upon the application in an electric field. At any pH range above the pI, the protein has a negative net charge and migrates toward the positive electrode. The opposite occurs at pH values lower than the pI where the positive net charge of the protein leads to movement toward the negative electrode. By using the appropriate buffer systems at a certain pH, proteins with different charges will migrate at different speed. Therefore, when a mixture of proteins is subjected to electrophoresis, each protein moves to a unique position in the electric field and separates from the others after a given length of time. This is because each protein molecule possesses a unique charge, size, and shape (Catsimpoolas, 1977; Cooper, 1977). The ionic groups on amino acids which have the greatest contribution to the charge of a protein are the carboxylic, amino, imidazole, sulfhydryl, phenoxy, and guanidinium groups (Catsimpoolas, 1977).

Electrophoresis is usually carried out in a porous supporting matrix, such as polyacrylamide or agarose gel. The pore size of the acrylamide gel can be controlled by varying the concentration of acrylamide in the gel. Protein molecules have to pass through the pores in the gel and the gel reduces protein migration by imposing a sieving effect. Therefore the mobility of a protein in acrylamide gels is a function of its net charge and its size (Catsimpoolas, 1977; Shuster, 1971).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Sodium dodecyl sulfate (SDS) dissociates protein into its constituent peptides by binding to the hydrophobic regions of the molecule and imparting a negative charge to the denatured protein (Cooper, 1977). At concentrations greater than the SDS monomer concentration of 0.02%, 1.4 g of SDS is bound to one gram of protein after reduction (Reynolds and Tanford, 1970). This high binding ratio will mask the native charge of most proteins and result in a situation in which there is a constant negative net charge per unit mass protein. The presence of carboxyl moieties in proteins, chemical modification of proteins, or the failure to reduce disulfide bonds can all result in interference of SDS binding to the protein and thus reduced electrophoretic mobilities.

Protein-SDS complexes behave as rod-like particles; the particle length varies as a the function of molecular weight of the polypeptide chain (Weber et al., 1972). Because the net charge per protein mass is constant, and the hydrodynamic properties are a function of the polypeptide length, the electrophoretic mobility of a protein becomes a function of its molecular weight. Weber and Osborn (1969) showed that the relative mobility of a protein is a linear function of the logarithm of its molecular weight. With 40 different well characterized proteins, they also proved that the molecular weight of proteins can be estimated accurately with only 3% deviation. Thus the molecular weight of a given protein can be determined by comparing its electrophoretic mobility to that of a marker protein with known molecular weight (Dunker and Rueckert, 1969; Weber and Osborn, 1969, 1975).

Isoelectric focusing. The isoelectric focusing (IEF) separation of proteins is carried out in a pH gradient established between two electrodes (Catsimpoolas, 1975, 1977; Chrambach and Baumann, 1976; Righetti and Drysdale, 1974; Rilbe, 1976). A pH gradient is formed after electrolysis of amphoteric buffer substances known as carrier ampholytes (Vesterberg, 1976). Amphoteric substances are charged molecules whose charges are dependent on the pH of the environment; positively charged at acidic pH and negatively charged at alkaline pH. During electrophoresis, there is a continuous production of H^+ and OH^- ions at the electrodes. Thus the pH increases at the cathode and decreases at the anode. The formation of such a pH gradient between the electrodes will then cause the carrier ampholytes to migrate to and concentrate within positions corresponding to their pI. Carrier ampholytes usually have a high buffering capacity at their isoelectric point (Svensson, 1961; Vesterberg, 1969). Therefore by using a series of different species of ampholytes, the pH gradient formed in the gel can be dictated.

Protein molecules, also being amphoteric (Vesterberg, 1971), focus in a similar manner as carrier ampholytes. When introduced into a pH gradient system, proteins will migrate to pH zones that correspond to their respective pI where their overall net charge is zero. Since proteins have variations in isoelectric points, they are focused at different regions of the pH gradient, resulting in their separation. The measurement of the pH at the focusing position provides a direct determination of the isoelectric point of the particular protein species.

After proteins are focused in a pH gradient, any movement of the proteins by diffusion away from the isoelectric pH causes restoration of electric charge and consequently electrophoretic movement toward the pI. By this counteraction of back-diffusion, separated protein molecules can be concentrated into extremely sharp bands with a resolution of 0.005 pH units or less (Righetti and Drysdale, 1974). This degree of resolution is superior to the conventional electrophoretic or chromatographic procedures.

Two-dimensional gel electrophoresis. The best protein separation can be achieved by high resolution two-dimensional gel electrophoresis (2-DGE) developed by O'Farrell (1975). This method involves the separation of proteins in the first dimension according to their charge and in the second dimension with SDS-PAGE according to their relative mobility. Because this involves two different protein separation mechanisms, the 2-DGE achieves a far better protein separation with higher resolution than any one-dimensional electrophoretic technique. O'Farrell (1975) has successfully separated over 1000 proteins from an E. coli lysate using 2-DGE. This technique has been widely applied, due to its high resolution to detect any changes in charge or size of molecules, for analysis of post-transitional modification (i.e., glycosylation, sulfation, or phosphorylation) of proteins (Dunbar, 1987), or to study gene expression in a cell or a tissue (Neal et al., 1984; Rabilloud et al., 1985).

A practical advantage of using 2-DGE is that this technique can simplify the characterization and purification of proteins for the development of monoclonal or polyclonal antibodies. The specific proteins separated by 2-DGE can be transferred to nitrocellulose paper and used for animal immunization. SDS is removed during the antigen preparation process. In addition, the recovery of the immunogen can be increased up to 100% (Chiles et al., 1987; Knudsen, 1985; Tracy et al., 1983).

Application of Electrophoretic Methods for Species Identification

The protein fingerprint obtained by electrophoretic methods for each species can be used for speciation (Lin and Lanier, 1980; Toom et al., 1983). However, the proteins are sometimes not completely separated. Thus the electrophoretic methods can only be applied for distal species comparison. The resolution of the electrophoretic protein patterns can be improved by incorporating SDS into the gel. The formation of the SDS-protein complex will reduce the hydrophobic protein interaction. Therefore, heat-denatured proteins can be well resolved by dissociating protein aggregates with SDS to reveal species differences. This then made it possible to identify the food components of heat-processed products. Using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), shrimp species, cultivars of Vicia fava beans, and varieties of wheat were identified as well as shrimp mixtures or wheat and rye mixtures (An et al., 1988; Barratt, 1980; Fullington et al., 1980; McCausland and Wrigley, 1976). The genus of boiled shrimp was identified and its content in mixture samples was determined after proper extractions were made from heat-denatured samples (An et al., 1988).

The use of a pH gradient greatly enhances the electrophoretic resolution of proteins and improves the effectiveness of this technique for species identification (Hamilton, 1982; Krzynowek and Wiggin, 1979, 1981; Lundstrom, 1980, 1981b, and 1983a and b). Electrophoretic patterns obtained by IEF have been extensively applied for speciation of fish (Hamilton, 1982; Lundstrom, 1980, 1981b, 1983a and b; Toom et al., 1983; Yamada and Suzuki, 1982). Fish, such as cod (Gadus callarias) and haddock (Melanogrammus aeglifinus), which could hardly be differentiated by polyacrylamide gel electrophoresis were properly identified by IEF (Toom et al., 1983). Using IEF, Lundstrom (1981b, 1983a) and Hamilton (1982) have successfully identified Pacific rock fish (Sebastes) and other fish species such as cod, haddock, pollock, cusk, red hake, red snapper, weakfish, American plaice and gray sole. Varieties of winged bean which could not be differentiated by SDS-PAGE were also identified by IEF (Westermeier et al., 1985).

Enzyme activity can be retained after focusing in nondenaturing IEF conditions. The presence of a particular enzyme can be detected by adding substrates to the solution in which the gel is suspended; the enzyme will convert the substrates to chromogenic compounds (Harris and Hopkinson, 1976). This process enables the detection of the enzyme of interest. Therefore, IEF can be successfully used to detect meat or fish species as well as contaminant species mixed at low concentrations in beef (King, 1984; King and Kurth, 1982; Sutton et al., 1983). Various species of fish including carp and trout were analyzed by enzyme staining the red cell superoxide dismutase, which showed polymorphism between various fish species (Sutton et al., 1983). When the IEF pattern was stained for adenylate kinase, phosphoglucomutase, and phosphogluconate dehydrogenase, the contaminant species (1%) such as sheep and goat in beef were detected (King and Kurth, 1982). By staining for these enzyme activities, the sheep and goat contaminants could be distinguished; which was not achieved by staining the total proteins. Species of cooked meats could be identified by staining for highly heat-stable enzymes, such as adenylate kinase and creatine kinase (King, 1984). These enzymes retained their activities after heating at 115° and 100°C, respectively, for 30 min.

They have been successfully used to differentiate the species among beef; chicken; buffalo; horse; kangaroo; and goat; as well as to detect their presence in beef.

Immunological Identification Methods

Immunological techniques such as the Ouchterlony test (Hayden, 1978, 1981) or a hemagglutination inhibition test (Karpas et al., 1970) have been used effectively for species identification and detection of adulterated meat. Rabbit antisera developed against heat-stable antigens of equine, porcine, ovine or avian adrenals or chicken troponine was proven to be effective for detecting the presence of these species in cooked beef sausage adulterated at a level of 1, 5 and 10%, respectively (Hayden, 1977, 1981). In an attempt to improve immunological techniques, many investigators proposed the use of enzyme-linked immunosorbent assays (ELISA) for species identification of raw meat or for determination of soya protein in food (Hitchcock et al., 1981; Kang'ethe et al., 1982; Patterson et al., 1984). Whittaker et al. (1983) listed the advantages of an ELISA method as follows: (1) the testing can be performed in approximately three hours, (2) smaller volume of species-specific antisera is required, (3) antisera can be mixed for simple screening procedures, (4) equipment is available to semi-automate the assay procedure and the recording and reporting of results, and (5) the increased sensitivity allows for use of simpler sampling techniques. In spite of the many advantages of this technique, cross reactivity occurred and posed a problem for the assay. Bovine, sheep, and horse-specific antisera each cross-reacted with buffalo, goat, and donkey meat, respectively (Kang'ethe et al., 1982; Patterson et al., 1984; Whittaker et al., 1983).

Principles of Immunological Assays

Precipitin reaction. Multivalent antigens may interact with multivalent antibodies to form large insoluble lattices of small soluble complexes and precipitate out of the solution. From the precipitation of the antigen-antibody complexes, the amount of antigen or antibody in a test sample can be estimated (Hood et al., 1984).

Ouchterlony double diffusion. Antigen-antibody precipitates can be observed by allowing one reactant to diffuse into a gelatinous medium, such as agar, that contains the other reactant. Antigen and antibody molecules placed in small wells cut into an agar slab diffuse out of their respective wells into the agar at a rate inversely proportional to their molecular weight. When an antigen reacts with an antibody, a line of precipitation is formed at where the two reactants diffuse together (Hood et al., 1984).

The Ouchterlony technique can be used to determine the identity of antigens; whether two antigens are different or identical, or whether they share some but not all antigenic determinants. The reaction of identity, partial identity, or nonidentity can be determined by the patterns of precipitin lines.

Agglutination. Cells can be agglutinated by cross-linking with antibodies. The cell agglutination technique is used to assay antibodies specific for antigens that either are normally present on a cell surface or can be coupled artificially to a cell surface (Hood et al., 1984). Agglutination technique is commonly used for the typing of human blood.

Immunoelectrophoresis. Immunoelectrophoresis provides improved resolution for complex samples by combining electrophoresis of antigens in one dimension with a subsequent double diffusion of antibodies and electrophoresed antigens in a second dimension (Hood et al., 1984). Serum is placed in wells near one side of a large slab gel and subjected to electrophoresis. Slots are then cut into the gel parallel to the direction of electrophoresis and filled with various test antibodies. The antibodies and the separated antigenic components diffuse toward each other through the gel and form precipitin lines when the specific antigenic components are reactive with the antibodies.

Indirect assays. Antigen-antibody complexes can be detected using indirect methods that utilize a secondary antibody in situations where labeling of neither the antigen nor the antibody is convenient (Hood et al., 1984). In these methods, such as ELISA and immunodot, antigen and antibody are allowed to react, and the antigen-antibody complexes are detected by use of a secondary antibody, a radiolabeled immunoglobulin-specific heterologous antibody that will bind to the primary antibody. As an alternative to radiolabeling, antibodies can often be coupled to active enzymes that catalyze conversion of a soluble specific reagent to an insoluble product. The formation of the products indicate the presence of the antibody. As another alternative, the secondary antibody can be labeled with a fluorescent ligand and the reactivity can be either detected by exposure to a light source of appropriate excitory wavelength or quantitatively assayed in a fluorimeter.

Western blotting. This method allows the separation of cognate protein antigen in a complex protein mixture. The components of the mixture are first electrophoretically separated on a polyacrylamide slab gel in the presence of SDS. The protein are then electrophoretically transferred from the gel to a nitrocellulose sheet, which is then incubated with antibody which has been conjugated with radioactive molecules. After being washed to remove free antibody, the membrane paper is dried, and the protein bands which reacted with the antibody are made visible by autoradiography. Alternatively the antigen-antibody complexes can be detected by use of a secondary antibody, a enzyme-labeled or fluorescent immunoglobulin-specific heterologous antibody that will bind to the primary antibody. This method detects antigenic determinants common to both the denatured and native forms of the proteins.

Principles of Hybridoma Technique

Humoral immunity in vertebrates is affected by antigen-binding antibodies that are secreted by cells of the B lymphocyte lineage (Alt et al., 1987; Tonegawa, 1983). B cells are differentiated by specific binding of antigen to the surface receptor of immunoglobulin present on the cell, and thus only the stimulated B cells are propagated into antibody-secreting plasma cells. Antibodies produced by a plasma cell have an identical specificity, but each plasma cell secretes antibodies with different specificity. Therefore, the immunization of an animal with complex antigen can cause the induction of B lymphocyte differentiation into plasma cells to produce polyclonal antibodies with various specificity. For the production of monoclonal antibodies (McAb), plasma cells responding to an individual antigenic determinant must be isolated for homogeneous specificity.

The technology devised by Kohler and Milstein (1975) permits the production of perpetually growing plasma cell lines which secrete antibodies of predefined specificity in large quantities. Hybridoma cell lines are produced by fusion of the immunized spleen cells with drug-resistant myeloma cells. Different types of cells can be made to fuse in the presence of fusogens, such as Sendai virus, lysolecithin, or polyethylene glycol (PEG) (Foster, 1982). The fusion of the plasma membranes results in the formation of heterokaryons which possess two or more nuclei. At the next cell division, the nuclei fuse and a hybrid cell results.

Fused cell culture arising from the fusion of tumor cells and normal cells can be rapidly overgrown by unfused tumor cells. To prevent this, a selection process is used to ensure that only hybrids will grow. The main biosynthetic pathways for purines and pyrimidines can be blocked by the folic acid antagonist, aminopterin (Goding, 1982; Littlefield, 1964). However, the cell can still synthesize DNA using thymidine kinase (TK) and hypoxanthine guanine phosphoribosyl transferase (HGPRT) enzymes via the salvage pathways in which the preformed nucleotides are recycled (Galfre and Milstein, 1983; Littlefield, 1964; Sinkovics and Dreesman, 1983; Zola and Brooks, 1982). DNA synthesis can still occur in those cells supplemented with thymidine and hypoxanthine, provided that the enzymes TK and HGPRT-are present (Fig. 1). When spleen cells are fused with myeloma cells lacking TK or HGPRT, only the hybrid cells can grow and propagate in a medium containing hypoxanthine, aminopterin and thymidine (HAT medium), because the spleen cells will provide the myeloma cells the necessary enzymes to synthesize nucleic acids by the salvage pathway. Fusion between spleen cells also occurs, but the spleen cell hybrids naturally die off within a week. Therefore, only hybrids arising from the fusion between myeloma and spleen cells are selected and actively multiply in the HAT selective medium. The growing hybrids are then subjected to limited dilution and individual clones that secrete the desired antibodies are selected (Zola and Brooks, 1982). Such antibodies are therefore of monoclonal origin;

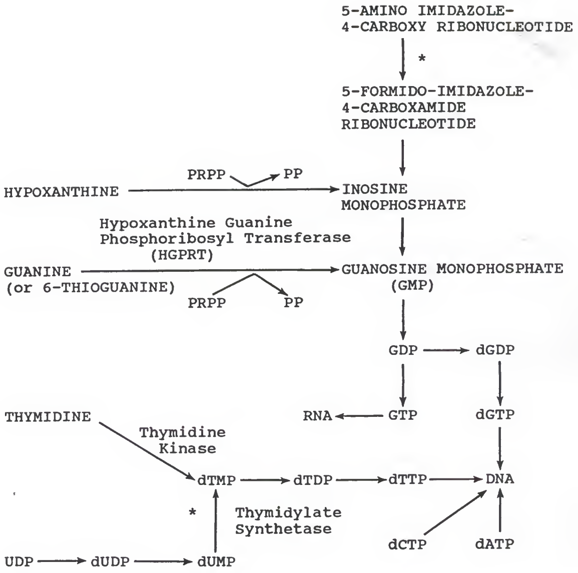


Figure 1. Metabolic pathways relevant to hybrid selection in medium containing hypoxanthine, aminopterin and thymidine (HAT medium). When the main synthetic pathways are blocked with the folic acid analogue aminopterin (*), the cell must depend on the "salvage" enzymes HGPRT and thymidine kinase. HGPRT- cells can be selected by growth in medium containing the toxic base analogues 6-thioguanine or 8-azaguanine, which are incorporated into the cell via HGPRT. Only HGPRT- cells survive. HGPRT- cannot grow in HAT medium unless they are fused with HGPRT+ cells. (Adapted from Goding, 1983)

and the selected clones, like ordinary myeloma lines, can be maintained indefinitely.

Monoclonal antibodies provide many advantages over conventional antisera (Edwards, 1981). All the molecules of monoclonal antibody are identical in amino acid sequence and binding properties. Thus, the antibody can have exceptional specificity and greatly reduced cross-reactivity which has been a common problem associated with polyclonal antisera. Monoclonal antibodies can also increase the sensitivity of the immunological assay. The monoclones will provide for an indefinite supply of species-specific antibody. Therefore, McAb could serve as an effective replacement for conventional antisera in identifying fish species or seafood species or to detect component species present in food products.

Application of Monoclonal Antibodies in Immunochemical Identification Techniques

The monoclonal antibody technique was first applied by Lunstrom (1984, 1985) to identify fish species and to differentiate fishery stock. McAbs were prepared from immunized mice with crude protein extracts of over 40 fish species. However, species-specified McAbs could not be found for any fish species. The numerous McAbs obtained this way showed a high cross-reactivity between unrelated species of fish, and one species showed a high reactivity with numerous unrelated McAbs (Lundstrom, 1984). In an attempt to utilize the antibodies, a species identification table was constructed in which species can be detected by a positive and negative reaction with the McAbs. However, no correlation was seen between the species of fish and the reaction pattern of the antibodies. It was speculated that most of the antibodies produced by the immunization with whole crude fish extracts might have specificity to the most prevalent proteins, such as albumin, rather than the species-specific proteins. Similar results were also reported by Tracy et al. (1983) who injected a protein extract of whole human urine into mice to produce monoclonal antibodies of various specificities. Most of the positive hybridoma cells produced antibodies to albumin, an abundant urinary protein in renal disease rather than the protein of interest, PC-30. The investigators then developed monoclonal antibodies with the antigens of interest separated by two-dimensional gel electrophoresis. The monoclonal antibodies obtained were specific for PC-30, the antigen used, as confirmed by autoradiography.

Monoclonal antibodies have also been used to detect components with high specificity in various food products (Brandon et al., 1987, 1988; Skerritt, 1985; Skerritt and Smith, 1985; and Skerritt et al., 1985). Brandon et al. (1987, 1988) showed that the use of McAb can be more sensitive and specific for antigen detection. Conventional antisera detected soybean trypsin inhibitor with less than 25% reactivity compared with that of monoclonal antibodies. Skerritt and his colleagues developed monoclonal antibodies by immunizing mice with wheat gliadin in order to detect the presence of gluten in various foods. The monoclonal antibody chosen by these investigators was successfully applied in baked goods and processed meats as well as natural food to give a quantitative and qualitative confirmation. The success of the study with heat processed products was thought to be due to the heat-resistance of gliadin which remained unchanged even after heat treatment for 100 min in water, as indicated by the electrophoretic patterns (Skerritt and Smith, 1985).

Chromatographic Identification Methods

Chromatography is widely used as a separatory analytical technique. The advantage of using high performance liquid chromatography (HPLC) over the use of other forms of liquid chromatography may be summarized as follows (Hamilton and Sewell, 1982): (1) the HPLC column can be used many times without the need to regenerate; (2) the resolution achieved on such columns far exceeds that of the other methods; (3) the technique is less dependent on the operator's skill due to high reproducibility; (4) the instrumentation of HPLC lends itself to automation and quantitation; (5) the analysis times are generally much shorter; (6) preparative liquid chromatography is possible on a much larger scale.

Principles of Chromatographic Methods

There are various separation methods available for HPLC. These include: liquid-liquid, liquid-solid, ion-exchange, ion-pair, exclusion, and affinity. The separation of analyte components results by the intermolecular interactions, such as Coulombic forces, dipole-dipole interactions, dipole-induced dipole interactions, ion-induced dipole interactions, dispersion forces, and hydrogen-bonding interactions, which occur between the stationary and mobile phases.

Liquid-liquid chromatography. Liquid-liquid chromatography (LLC) involves the use of a liquid mobile phase and a liquid stationary phase which is either coated onto a finely divided inert support (partition chromatography) or chemically bonded (bonded phase chromatography) to the support material (Johnson and Stevenson, 1978). The sample to be analyzed is dispersed in the mobile phase and its components are partitioned between the stationary and the mobile phases according to their partition coefficients (Johnson and Stevenson, 1978). This partitioning leads to a differential rate of migration for separation to occur.

There are two phases available for liquid-liquid chromatography: normal phase and reverse phase. Normal phase liquid-liquid chromatography originally referred to a system with a polar liquid stationary phase, e.g., water, glycol, or nitrile, and a relatively non-polar mobile phase, e.g., hexane, benzene, or chloroform (Pryde and Gilbert, 1979). This mode of operation was used to separate polar compounds which would distribute preferentially in the polar stationary phase. A system with a non-polar stationary phase, e.g., a hydrocarbon, and a polar mobile phase, e.g., water, is referred to as reverse-phase liquid chromatography (Pryde and Gilbert, 1979). In reverse-phase chromatography the non-polar stationary phase may be displaced from a polar support by virtue of the preferential adsorption of the polar mobile phase. Recently the term "reverse phase" chromatography has been redefined. Reverse phase now refers to a system where the stationary phase is less polar then the mobile phase (Hamilton and Sewell, 1982).

Liquid-solid (adsorption) chromatography. In liquid-solid chromatography (LSC), the separation is carried out with a liquid mobile phase and a solid stationary phase which reversibly adsorbs the solute molecules (Johnson and Stevenson, 1978). The stationary phase may either be polar or nonpolar depending on the type of the mobile phase used. The retention of samples in LSC is more predictable than in LLC, since the elution order follows the polarity of the solutes (Hamilton and Sewell, 1982). This method is more sensitive to compound type than LLC but less sensitive to molecular weight differences between the solute species (Hamilton and Sewell, 1982).

Ion-exchange chromatography. Ion-exchange involves the substitution of one ionic species for another. The stationary phase consists of a rigid matrix; the surface of which carries a net positive or negative charge to give an ion-exchange site (Regnier, 1982). When a mobile phase containing anions is used, the positive exchange site will attract and hold a negative counter-ion. Anion samples will then exchange with the negative counter-ions. Since the process involves the exchange of anions it is known as "anion exchange" (Johnson and Stevenson, 1978). The complementary process of "cation exchange" occurs when the stationary phase surface carries a net negative charge. The positively charged counter-ions will then be exchanged with positively charged sample ions.

The separation is based on the strength of the interactions between the sample ions and the exchange site. Ions that interact only weakly with the exchange site will be poorly retained and will have small chromatographic capacity factor values (k', the ratio between the number of moles of solute in the stationary and mobile phases), while ions that have strong interactions will be strongly retained and will have high k' values (Pryde and Gilbert, 1979).

Ion-pair chromatography. Ion-pair chromatography can be used to separate both ionic or ionisable molecules (Kealey, 1987). An ionic or ionisable sample too polar to interact with the apolar hydrocarbon phase is rendered less polar by adjusting the pH and/or adding an ion-pairing agent. The resultant unionized or ion-pair molecule is hydrophobic in character and will be retained by the column due to the interaction with the hydrocarbon stationary phase.

Exclusion chromatography. Exclusion chromatography is also referred to as gel filtration, gel permeation chromatography, or gel chromatography. Exclusion chromatography separates substances according to their molecular size and shape. Small molecules that can enter freely into the pores of the stationary phase are said to have a distribution coefficient of K=1, and large molecules which are completely excluded from all pores have a distribution coefficient of K=0, while molecules of an intermediate size will have distribution coefficients between 0 and 1. Thus large molecules with lower K values will move more rapidly through the column than will the smaller molecules, and be eluted first. Molecules are therefore eluted in order of decreasing molecular size (Hamilton and Sewell, 1982; Lazure et al., 1982).

Affinity chromatography. Affinity chromatography utilizes the unique biological specificity of the interaction between a protein molecule and a suitable ligand (Kealey, 1987). The ligand with a specific binding affinity for the protein is covalently bonded to a gel matrix to form a stationary phase. Upon the addition of sample molecules the protein components with a specific affinity for the ligand are bound and retained. Unbound material is eluted from the column in the mobile phase, and the bound proteins can be released by changing pH of the mobile phase.

Application of Chromatographic Methods in Species Identification

Reverse phase HPLC has been widely used for protein separation for various purposes, such as identification of fish species, and characterization of muscle proteins or metmyoglobin after heat denaturation (Davis and Anderson, 1984; McCormick et al., 1987; Oellingrath, 1988; Osman et al., 1987). In a study using HPLC to analyze the water-soluble proteins from 31 fish species, Osman et al. (1987) showed that based on the qualitative and quantitative differences of the chromatograms, species identification could be achieved. They also showed that the freezing of whole fish up to 6 months did not have any apparent effect on the chromatograms; however, cooking caused significant changes of the chromatograms. Thus, HPLC could not be used to identify the species origin of processed or heat-treated fish samples.

McCormick et al. (1987) demonstrated the effect of thermal treatment on the HPLC chromatograms of muscle sarcoplasmic proteins. Thirteen fresh porcine muscle proteins were separated by HPLC. However, only 3 remained soluble after the muscle extracts were heated to 70°C. They were identified to be myoglobin, pyruvate kinase, and lactate dehydrogenase.

Shrimp Species Identification by Conventional Method

The conventional method for shrimp species identification is based on morphology variation of the various parts of the body, such as carapace, abdominal segments, antennae, telson, orbit, eyestalk, rostrum, etc. (Fig. 2). The identification process is constructed by dichotomous keys for easy use according to the morphological characteristics of animals (Williams, 1984). A brief description of the morphological characteristics of pink, white, and rock shrimp used in this study is made from the book, "Shrimps, Crabs, Lobsters of the Atlantic Coast" (Williams, 1984).

The pink shrimp (*Penaeus duorarum*) may be recognized by the following characteristics listed below. The dorsal margin of the rostrum is slightly arched. The median carina on the carapace has a deep median groove, which is sometimes widened

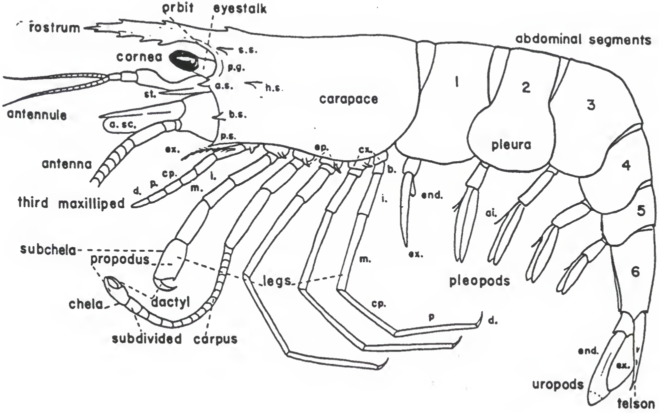


Figure 2. Schematic drawing of shrimp in lateral view: ai, appendix interna; as, antennal spine; asc, antennal scale; b, basis; bs, branchiostegal spine; cp, carpus; cx, coxa; d, dactyl; end, endopod; ep, epipod; ex, exopod; hs, hepatic spine; i, ischium; m, merus; p, propodus; pg, postorbital groove; ps, pterygostomian spine; ss, supraorbital spine; st, stylocerite. (Adapted from Williams, 1984)

at the anterior or posterior half. Thelycum of the female has broad lateral plates contiguous mesially or overlapping anteromesially, covering a rather small posterior projection or median protuberance. The anterior process bounds a central concavity with a subtriangular ridge. Petasma of the male has rather long distomedian projections extending free over the ventral costae; the latter are unarmed along the distal border but with 6 to 12 pointed teeth in 2 irregular rows on the attached border. The distal fold forms a large rounded auricle armed with numerous spines in a half-moon formation on the inner surface. The ventrolateral lobule is armed externally with spines increasing in number proximally.

White shrimp (Peneaus setiferus) is recognized by its morphological characteristics listed as follows. The white shrimp has a thin, polished, and translucent integument. The carapace has a continuous median carina anteriorly with the rostrum and extends backward about 2/3 length of the carapace. The carapace is not grooved posteriorly, and the lateral rostral grooves terminate near the posterior tooth in roastral series. The carapace is armed dorsally with 5-11 sharp teeth with anterior teeth 6 on rostrum with the gastrofrontal carina absent. The rostrum has a long, slender, unarmed, and distally up-curved tip. The first dorsal tooth is near the distal edge of the eye. The ventral edge is usually armed with 2 teeth. The antennal spine extends upward a short distance from the hepatic spine. The mesial antennular flagellum of an adult male is flattened dorsoventrally and armed dorsally with teeth. The antennal flagellum is very long.

Rock shrimp (Sicyonia brevirostris) are characterized by the features listed as follows. The rostrum is variously curved dorsally slender, and narrowing considerably to tip. The rostrum is armed dorsally with 2 or 3 subequal teeth (not counting bifid or trifid tip) with a ventral tooth projecting anteriorly farther than the dorsal teeth. A small node is often present between the terminal teeth. High postrostral carina has 3-4 teeth behind orbital margin. The 3 large teeth are behind the hepatic spine and front of a small tooth. The antenna is armed with small buttressed spine. The hepatic spine of rock shrimp is well developed, and ocular stylets are long. The abdomen has prominent tergal carina deeply notched posteriorly on the first 5 segments. Segment 1 has carina produced into an elevated tooth directed anteriorly with conspicuous teeth on posterior margin of segments 5 and 6. The ventral margins of the pleura are bordered by a smooth and narrow ridge. The margins of the first 3 segments are broadly rounded posteriorly, but the segment 4 is rectangular or spined in shape. The anteroventral angle of segments 1-4 is armed with a spine, which is progressively more developed, and is curved laterally or lateroposteriorly and dorsally in series. Segments 5 and 6 are broadly rounded anteriorly, and the posterolateral angle has acute tooth. The anterior margin of the first segment and the posterior margins of segments 1-3 are slightly concave. A deep V-shaped indentation is near the midpoint of the posterior pleural margins on segments 4 and 5. Segment 6 has a pronounced angular projection on the posterior margin near the midpoint. The anterior and posterior tergals are well defined. The posterior pleural is shallow. Segments 4 and 5 are traversed by an anterior tergal terminating near the midpoint. The posterior tergal is long and continuous to curve anteriorly near the ventral margin of pleuron on segment 4 only. Segment 6 has a shallow and short with broad longitudinal sulcus. The sulci are on segments as follows. The segment 1 has a deep anteromedian pleural dorsally terminating at the concavity of tergal margin. A long and well-defined posteromedian continues to the dorsal side of tergum. The posterior tergal is shallow and often obscure. The posterior pleural is also shallow.

CHAPTER III
ELECTROPHORETIC IDENTIFICATION OF RAW AND COOKED SHRIMP USING VARIOUS PROTEIN EXTRACTION SYSTEMS

Introduction

Willful or unintentional adulteration by substituting lower quality and/or lower priced seafood products for higher priced products has led to an increasing demand for identifying species of fish or other seafood in the marketplace. There are at least three broad testing methods available for species identification: chemical, electrophoretic and immunological methods (Kurth and Shaw, 1983). Several official electrophoretic methods, such as starch gel-zone electrophoresis, acrylamide disc electrophoresis, thin layer polyacrylamide gel isoelectric focusing and cellulose acetate strip, have been approved by AOAC (1984) to differentiate species of seafood or seafood products. By using isoelectric focusing, Lundstrom (1981b, 1983a) and Hamilton (1982) have identified successfully Pacific rock fish (Sebastes) and other fish species such as cod, haddock, pollock, cusk, red hake, red snapper, weakfish, American plaice and gray sole. The highly water-soluble sarcoplasmic proteins consisting of glycolytic enzymes, myoglobin and other proteins present in the intracellular fluid of muscle (Goll et al., 1977; Scope, 1970) were often used for species identification (Lundstrom, 1981b, 1983a;

Hamilton, 1982). However, very little information exists on the relative effectiveness of various solvent systems in extracting proteins for gel electrophoresis.

Protein solubility is affected by several factors, such as pH, ionic strength, temperature or organic solvents (Thakker and Grady, 1984). Solutions of 1% sodium dodecyl sulfate (SDS), 8 M urea, 0.1 M NaCl and a water homogenate adjusted to pH 8.0 have all been used to effectively extract soluble proteins for biochemical or electrophoretic studies (Chang and Sosulsky, 1985; Dunker and Rueckert, 1969; Krzynowek and Wiggin, 1979, 1981; Regenstein and Stamn, 1979; Tanford, 1968; Thakker and Grady, 1984; Tuszynski et al., 1978).

Solubility of sarcoplasmic proteins was affected significantly after heat treatment. Blue crab meat (Callinectes sapidus) heated at 100°C for 5 min caused a 90% decrease in extraction of water-soluble proteins compared to meat treated at 40°C for the same length of time (Dowdie and Biede, 1983). Heat treatment reduced the water-extractable proteins as demonstrated by comparing electrophoretic gels of heated to non-heated samples (Caldironi and Bazan, 1980; Dowdie and Biede, 1983; Koohmaraie et al., 1984; Yowell and Flurkey, 1986). The solubility of heat-denatured proteins can also be improved by using various solvent systems such as urea and SDS (Tanford, 1968; Thakker and Grady, 1984). A 10 M urea solution and thin layer gel isoelectric focusing was successfully used to identify the various genus of cooked and frozen crab meat (Krzynowek and Wiggin, 1979, 1981). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) has been utilized to separate proteins and to estimate their molecular weights (Weber and Osborn, 1969, 1975).

The objectives of this study were to evaluate the effectiveness of 5 solvent systems in identifying three species of raw and cooked shrimp; to use the best solvent(s) to check applicability of identifying shrimp species and their relative contents in fabricated samples by a blind study; and to examine the addition of protease inhibitor on SDS-PAGE protein patterns.

Materials and Methods

Shrimp Samples

Three shrimp samples, pink (*Penaeus duorarum*) (Key West, FL), white (*Penaeus setiferus*) (Jacksonville, FL), and rock shrimp (*Sicyonia brevirostris*) (Port Canaveral, FL) were harvested and transported within 48 hr to the Food Science and Human Nutrition Department, University of Florida, Gainesville. The three species were stored at -33°C until needed. They were thawed under tap water, peeled, and deveined. For the cooking treatment, shrimp muscle was placed in boiling water for 5 min.

Protein Extraction and Sample Preparation

Five solvents were compared for their effectiveness in extracting sarcoplasmic proteins from whole raw and cooked shrimp. They were water, water homogenate adjusted to pH 8.0 with 0.1 N NaOH, 0.1 M NaCl, 1% SDS (w/v), or 8 M urea. All five solvents contained 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM EDTA and 0.01% (w/v) sodium azide to inhibit proteases and microbial growth. Shrimp samples were chopped, mixed in a beaker with each solvent at a ratio of 1:3 (w/v), and homogenized using a Polytron (setting 5.5, Brinkmann Instrument, Westbury, NY) at room temperature for 1 min. The samples were then centrifuged at 48,000 x g for 20 min at 20°C, the supernatants were collected, and protein contents determined by Lowry method (Lowry et al., 1951). The presence of SDS (0.01%) or urea (0.08 M) in the diluted sample did not interfere with the Lowry method.

Protein was determined for the supernatants of water, 1% SDS or 8 M urea extract. The percent yield was calculated as the ratio of total protein content in the supernatant to that in the homogenate.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the modified procedure of Laemmli (1970) and O'Farrell (1975) using a Protean II (vertical slab) unit (Bio-Rad, Richmond, CA). Slab gels consisted of a running gel (10.4%) which was polymerized overnight, and a stacking gel (3.1%) which was poured 2 hr before sample application. Protein contents of the supernatants were adjusted to 1.75 $\mu g/\mu L$ with Tris-HCl buffer (pH 6.8) and SDS solution, then heat-denatured and run on SDS-PAGE (O'Farrell, 1975) after adding sucrose and bromophenol blue. A constant current of 15 mA/slab was applied initially and increased to 30 mA/slab when the marker front reached the running gel. Following electrophoresis, the proteins were stained with 0.125% (w/v) Coomassie Brilliant Blue R-250 (Bio-Rad) in 40% ethanol and 7% acetic acid, and then destained in 7% acetic acid. Electrophoretic patterns were recorded either by drying the gel using a slab gel dryer (Hoefer Scientific Instrument, San Francisco, CA) or by developing the positive image using a Kodak Electrophoresis Duplicating Paper (EDP paper, Eastman Kodak, Rochester, NY). Molecular weights of the protein bands were determined according to the method of Weber and Osborn (1969) and Weber et al. (1972) using a low molecular weight (MW) protein kit (Pharmacia, Piscataway, NJ) containing: phosphorylase b (MW, 94 kD), bovine serum albumin (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), soybean trypsin inhibitor (21.1 kD) and α-lactalbumin (14.4 kD). α-Lactalbumin was not included for MW determination due to its high R_f value (>0.90) (Dunker and Rueckert, 1969; Weber and Osborn, 1975).

Effect of Storage and Dilution of 8 M Urea Extracts on Protein Banding Patterns

Raw samples of pink, white and rock shrimp were extracted with 8 M urea containing PMSF as previously described. The supernatants were stored at 4°C for 4 hr and 1, 2, 4 and 6 days before determining protein. The samples were diluted to the desired protein concentration (35 μg/20 μL) and run on SDS-PAGE.

To characterize the effect of short-term exposure to 8 M urea on protein patterns, rock shrimp homogenate was run on SDS-PAGE at 1 hr and 4 hr after sample homogenization. A 4 hr exposure time was chosen because the total extraction/protein determination time was 4 hr. The 1 hr samples were prepared for electrophoresis based on the results from previous studies. The actual amount of protein applied was 1.62 μg/μL.

A study was also conducted to determine whether diluting the 8 M urea in the rock shrimp supernatant to 0.4 M (20-fold dilution) would help preserve the original protein patterns. In this diluted sample, protein was 1.75 μg/μL. It was stored at 4°C for 24 hr before running SDS-PAGE.

Effect of Protease Inhibitor on Protein Pattern

Pink shrimp proteins were extracted with water and the homogenate was left at room temperature for 0, 5, 10 and 24 hr. A reference group was extracted in the presence of 0.1 mM PMSF, 10 mM EDTA and 0.01% sodium azide and also allowed to stand for the same time periods. Both samples were analyzed by SDS-PAGE.

Blind Study

A blind study was performed on fabricated mixtures of pink and rock shrimp to determine the effectiveness of the various extraction methods along with SDS-PAGE in determining the presence of a specific species (rock shrimp) and its content in the mixture. Raw pink and rock shrimp were individually chopped and then mixed in various ratios based on fresh muscle weight. These ratios were unknown to the investigators. The mixtures were extracted with water. For heat (boiled) treatment, the chopped shrimp mixture was boiled for 5 min in a 2 oz Whirl-Pak bag (Fisher Scientific) and homogenized in 1% SDS. Following centrifugation and protein determination, the diluted supernatants were run on SDS-PAGE. The protein patterns were compared to reference samples of pink and rock shrimp at various ratios. The percent rock shrimp in the unknown sample was determined by visual estimation of the intensity of the species-specific protein bands.

Results and Discussion

Comparison of the Five Protein Extraction Solvents

The five protein extraction systems used in this study can be categorized into two general groups: non-denaturing and denaturing agents. Characteristic SDS-PAGE patterns were obtained for all the extracts of the raw and boiled shrimp. Only the patterns for white shrimp are shown (Figs. 3 and 4).

For all raw samples, a low ionic strength salt solution (0.1 M NaCl) or the adjustment of the homogenates to pH 8.0 did not increase protein extractability (data not shown) or change drastically the electrophoretic patterns when compared to the water extract (Fig. 3). Two common protein-denaturing solubilizers, SDS and urea, showed increased protein bands (Fig. 3).

Protein in the water extracts of raw samples was only 1/3 to 1/4 that of 1% SDS or 8 M urea extracts (Table 1).

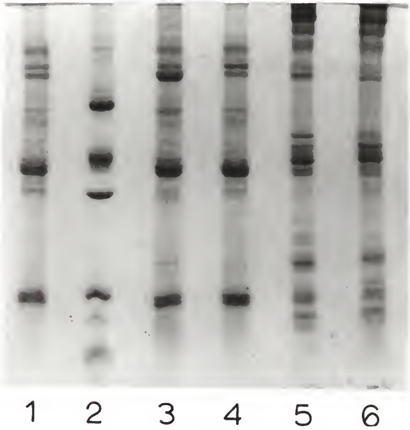


Figure 3. SDS-PAGE patterns of raw white shrimp extracted with water (1), water homogenates adjusted to pH 8.0 (3), 0.1 M NaCl (4), 1% SDS (5), and 8 M urea (6). Protein standards are also included (2).

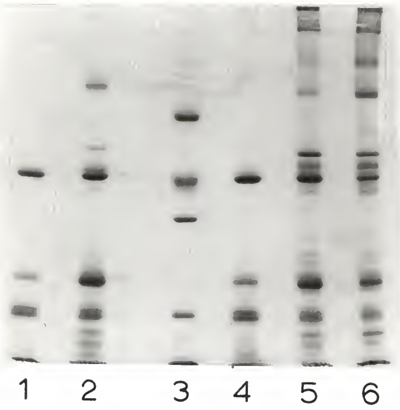


Figure 4. SDS-PAGE patterns of cooked white shrimp extracted with water (1), water homogenates adjusted to pH 8.0 (2), 0.1 M NaCl (4), 1% SDS (5), and 8 M urea (6). Protein standards are also included (3).

Table 1. Protein of the various shrimp extracts

Extraction System	Protein concentration (mg/mL)[a]					
	Pink shrimp		White shrimp		Rock shrimp	
	Raw	Boiled	Raw	Boiled	Raw	Boiled
Water	11.1±1.1	2.37±0.3	12.0±2.5	2.58±0.2	9.84±1.0	2.32±0.0
1% SDS	41.2±2.5	7.29±0.3	38.1±5.0	6.47±0.4	29.6±4.2	4.70±1.0
8 M Urea	45.0±1.5	20.0±0.9	38.3±6.2	19.3±2.1	37.7±2.7	19.2±0.6

[a] Protein content was determined by the Lowry assay. Data are means ± standard deviations from at least three expriments.

Low protein recovery was also noted for water extraction (Table 2). However, water was the best solvent to extract proteins from raw shrimp for electrophoresis. Specific patterns were obtained for each species, and distinct bands could be recognized and used for species differentiation. Water has been the most common solvent used to extract fish proteins for gel electrophoresis (Lundstrom, 1981b, 1983a).

Although protein in 1% SDS extracts of raw samples was not as high as those in 8 M urea extracts (Table 1), a higher recovery was noted for 1% SDS (Table 2). SDS produces a net negative charge on protein molecules and interacts with hydrophobic regions of proteins, thus separating them into subunits (Cooper, 1977). In addition, SDS also solubilizes membrane proteins. These contributing factors might be responsible for the higher recovery and the presence of more bands with high and low MW than water extracts (Figs. 3 and 4).

Urea was the most effective agent to dissolve proteins from both raw and boiled shrimp. Urea extracts gave the highest protein content (Table 1). Protein bands with high MW were present (Figs. 3 and 4). However due to its high density, it gave a lower recovery than 1% SDS in extracting shrimp proteins. Urea at high concentration is known to reduce hydrophobic interactions and hydrogen bonding in proteins and will, therefore, cause the dissociation of molecules (Cantor and Schimmel, 1980; Dunker and Rueckert,

Table 2. Protein recovery of water, 1% SDS and 8 M urea in extracting shrimp protein[a]

Extraction System	Pink shrimp		White shrimp		Rock shrimp	
	Raw	Boiled	Raw	Boiled	Raw	Boiled
Water	8.9±3.0	7.8±1.8	23.1± 1.1	8.3±2.5	26.0±2.1	10.9±2.1
1% SDS	80.7±5.2	27.1±3.8	82.7± 5.0	47.7±2.0	73.3±4.4	17.3±3.5
8 M Urea	77.1±8.1	17.1±1.7	68.2±10.2	22.9±0.8	67.3±3.3	29.1±0.6

[a] Percentage recovery was calculated as the ratio of total protein content in the supernatant to that in the homogenate. Data are mean ± standard deviation from at least three separate experiments.

1969) which may contribute to the presence of some bands with low MW (Figs. 3 and 4) that are not found in water extracts.

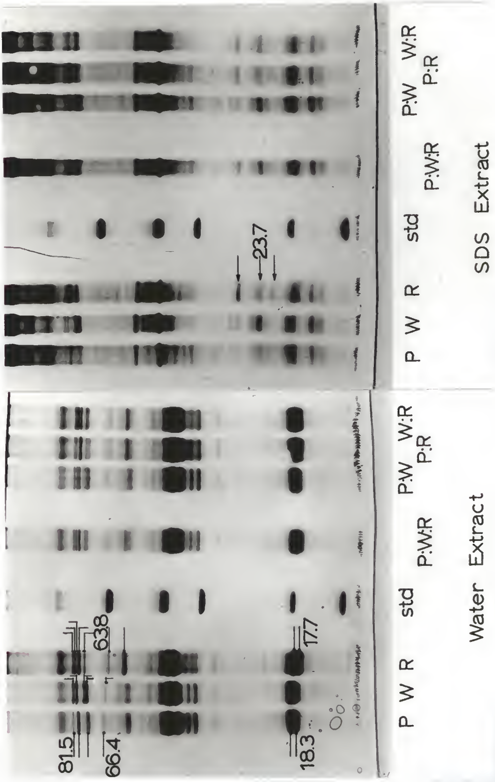
Heat treatment followed by water extraction resulted in a drastic change in SDS-PAGE patterns; the number of bands were reduced greatly as compared to raw samples (Fig. 4). Reduction in band numbers was also observed by Dowdie and Biede (1983) in heated blue crab meat. Concomitant reduction of protein bands from six to one was observed by Lee et al. (1974) as the processing temperature of bovine muscle was increased from 65° to 90°C. High temperature thus caused both denaturation and dissociation of subunits. Thus, water was not effective in extracting proteins from cooked shrimp. A protein yield of only 8-11% was obtained (Table 2). Protein from water extracts of the three boiled shrimp was about 25% of those of fresh shrimp (Table 1). The use of SDS or urea enhanced protein extractability (Table 2) and provided greater information for species identification (Fig. 4). The patterns of these two extracts were similar (Fig. 4), so SDS was chosen as the solvent for genus identification of cooked shrimp.

Species-Specificity of the SDS-PAGE Pattern

Characteristic gel patterns were obtained for all shrimp samples. For ease of comparison, the gel was divided into three equal segments, A (top), B (middle) and C (bottom).

Water extracts of raw samples showed the most diversity between species (Fig. 5a). Four major bands with MW of 88.3, 81.5, 78.6 and 74.1 kD were found in section A of pink shrimp while white shrimp had five bands with MW of 88.6, 81.7, 79.9,

Figure 5. SDS-PAGE patterns of water (a) and SDS (b) extracts of raw pink, white and rock shrimps and their mixtures (1:1 protein ratio). The numerical values are molecular weights of the specific proteins. P: pink, W: white and R: rock shrimp.


81.5
66.4
63.8
18.3
17.7
P W R std P:W:R P:W P:R W:R
Water Extract
23.7
P W R std P:W:R P:W P:R W:R
SDS Extract

77.7 and 75.7 kD in this section. Rock shrimp had six bands in section A with MW of 88.9, 82.1, 80.6, 79.5, 78.1 and 76.0 kD. A species-specific minor band was also found between section A and B. Its MW was 66.4, 65.7 and 63.8 kD for pink, white and rock shrimp, respectively.

Unlike those found in pink and white shrimp, the protein pattern for section B of rock shrimp were different. A minor intensity band with MW of 39.5 kD was only found in rock shrimp. Two major thick bands were detected in section C with MW of 19.1 and 18.3 kD for pink and white shrimp or 18.5 and 17.7 kD for rock shrimp. The two rock shrimp bands always migrated further than those from pink and white shrimp; they can be used for species-specificity identification.

Different banding patterns were observed for the SDS extracts of raw shrimp (Fig. 5b). Differences in intensities and minor banding patterns existed between pink and white shrimp. However, discernible differences were found only in section A and B for pink and white shrimp. The 78.6 kD band was only found in section A of pink shrimp. Two bands (80.5 and 76.7 kD) in section A and one band (41.1 kD) in section B were only present in white shrimp while rock shrimp had the greatest differences from the other two shrimp in section C. Three unique bands of 26.5, 23.7 and 21.5 kD were found in rock shrimp. The band 58.4 kD in section B was also unique for rock shrimp. The urea extracts of the three shrimps showed similar patterns as the SDS extracts.

In contrast to raw samples, the species-specificity of the water extracts of boiled shrimp was not easily discernible. Only a few bands were detected, and they were basically similar for all three species (data not shown). However, improved resolution was achieved for the SDS extracts (Fig. 6). Pink shrimp was found to have similar patterns as white shrimp, possibly because they belong to the same genus. Two characteristic thick bands (79.1 and 75.9 kD) found in section A of the rock shrimp extract were not as apparent as in white or pink shrimp. Variations were found in section C of rock shrimp as compared to the other two shrimp. Bands with MW of 27.7-31.3 kD were not present in rock shrimp, while three new bands of 21.6, 18.7 and 17.8 kD were detected. A new band with a MW of less than 27.7 kD was also found in rock shrimp. Again, urea extracts of boiled shrimp revealed the same patterns as the SDS extracts. Distinct differences in banding patterns could only be differentiated between white or pink and rock shrimp.

Comparison of SDS-PAGE Patterns of Mixed Shrimp Protein Extracts

Characteristic banding patterns of each species were used in identifying the existence of that specific species in shrimp mixtures. For example, the 79.9 kD band of water extracted raw white shrimp (Fig. 5a) could serve as an important indicator for the presence of white shrimp in a white-rock shrimp mixture. The minor band specific for each species (66.4, 65.7 or 63.8 kD for pink, white or rock shrimp,

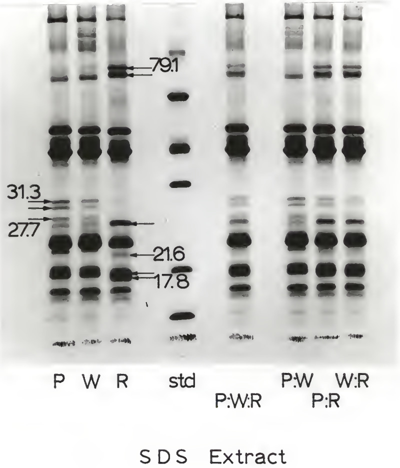


Figure 6. SDS-PAGE patterns of SDS extracts of cooked pink, white and rock shrimp and their mixtures (1:1 protein ratio). The numerical values are molecular weights of the specific proteins. P: pink, W: white and R: rock shrimp.

respectively) provided another useful indicator for species identification. The minor band of the pink and rock shrimp is resolved enough to distinguish these species (Fig. 5a). However, the minor band (65.7 kD of white shrimp) can overlap the minor band of rock shrimp, so great care is needed when using this band to identify rock and white shrimp mixtures. The 58.3 kD band of the rock shrimp (Fig. 5a) provides additional information on the presence of rock shrimp in a mixture. This sharp band is intense and can be easily recognized despite the presence of other bands with similar MW's (56.5 and 57.0 kD for the pink and white shrimp, respectively).

The SDS extracts of boiled shrimp demonstrated a genus-specific pattern (Fig. 6). The presence of two characteristic thick bands (79.1 and 75.9 kD) in section A, the 26.7 kD band in section B, and the 21.6 and 17.8 kD bands in section C would indicate the presence of rock shrimp in a mixture of sample. The 26.7 kD band is characterized by its intensity in mixture samples. The 21.6 and 17.8 kD bands are unique because they are only found in the SDS extract of rock shrimp.

The urea extracts of boiled shrimp proteins also showed similar gel patterns as those of the SDS extracts except for the presence of some minor bands in section B. The presence of rock shrimp in the mixture can be determined by the presence of two characteristic bands (27.1 and 22.0 kD). The 22.0 kD band was only detected in rock shrimp (data not shown).

Blind Study

Using SDS-PAGE, various extraction systems, and the information obtained in the previous studies, all unknown samples were correctly identified. The adulterated samples were detected at a maximum of 10% difference from what was actually present for both raw and boiled shrimp samples (Table 3) as judged by direct inspection using the 20% increment ratio for the mixed extracts as a reference.

Effect of Protease Inhibitor on Protein Banding Patterns

Proteolytic enzyme activities such as acid or alkaline proteases were found in the sarcoplasmic fractions of Atlantic croakers as well as in the liver, kidney and alimentary canal (Su et al., 1981). At physiological pH, both acid and alkaline proteases contributed to the proteolysis of fish products during processing at 45°-60°C (Lin et al., 1980). The activities of heat stable alkaline protease in the fish muscle of Pacific whiting even increased at 25°C or above with storage time (Erickson et al., 1983). Thus, the presence of these enzymes in the extract will affect the protein patterns. In this study, changes in electrophoretic patterns occurred when PMSF was not incorporated. The 74.1 kD band was not found in those samples lacking PMSF. However, a new band (59.3 kD) which broadened as the incubation time increased was formed (Fig. 7). The 74.1 kD protein is therefore susceptible

Table 3. The efficiency of the SDS-PAGE assay[a] in determining the percent content of rock shrimp in various mixtures by a blind study

Experiment	Actual rock shrimp content[b]	Detected rock shrimp content[c]	Difference[d]
Raw sample I			
	10	0	-10
	75	80-90	5-15
	30	20	-10
	50	40	-10
Raw sample II			
	10	10	0
	20	10-20	0-10
	50	40	-10
	30	20	-10
	15	10-20	+5
	5	<10	5
Cooked sample I			
	10	<10	0
	50	50	0
	5	<10	5
	20	30	10
	15	20	5
	30	20	-10
Cooked sample II			
	15	10	-5
	35	20	-15
	50	30	-20
	10	0-5	-5
	25	15	-10
	70	60	-10

[a] Water and 1% SDS were used to extract raw and cooked shrimp proteins, respectively.

[b] Actual rock shrimp content was the percentage of the shrimp in the rock-pink shrimp mixture prepared unknown to the investigator.

[c] Detected rock shrimp content was the percentage value determined by investigator after comparing the SDS-PAGE patterns of the unknown samples to standards which contained different amounts of rock shrimp in the rock-pink shrimp mixtures.

[d] Difference was derived by substracting the value of actual rock shrimp content from the detected values.

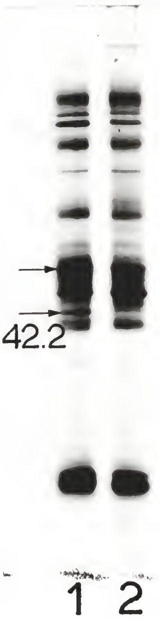


Figure 7. SDS-PAGE patterns of pink shrimp extracted in the presence (1) and absence (2) of protease inhibitor, PMSF. The numerical values are molecular weights of the specific proteins. P: pink, W: white and R: rock shrimp.

to proteolysis, and the addition of protein inhibitor (PMSF) is recommended. Currently, no attempt was made to control proteolysis in the AOAC (1984) protein extraction methods.

Effect of 8 M Urea Treatment on Protein Patterns

A 10 M urea extract used on the same day of preparation was recommended by Krzynowek and Wiggin (1979) to ensure the reproducibility of the protein fingerprints obtained by isoelectric focusing. Low reproducibility of patterns from urea extract was encountered when extraction was not performed on the same day as SDS-PAGE. Smeared patterns were observed with pink and rock shrimp. Therefore, studies were conducted to identify the proper procedures to handle urea extracts to minimize distortion.

A time-dependent degradation of proteins in 8 M urea extracts of raw shrimp stored at 4°C was noticed from changes in the SDS-PAGE patterns (Fig. 8). Protein bands of 114 and 99.3-74.1 kD in section A, the 50.3 kD in section B, and the 23.8, 20.1, 19.4 and 17.1 kD in section C for the pink started to disappear while a new band of 35.2 kD was formed. Protein degradation started to take place on day two of storage at 4°C for pink shrimp. The band of 35.2 kD also started to degrade within one or two days after its formation. Rock shrimp showed the most serious smearing problem (data not shown). The 35.2 kD band appeared on the second day after protein extraction. Consequently, only a large smeared band was

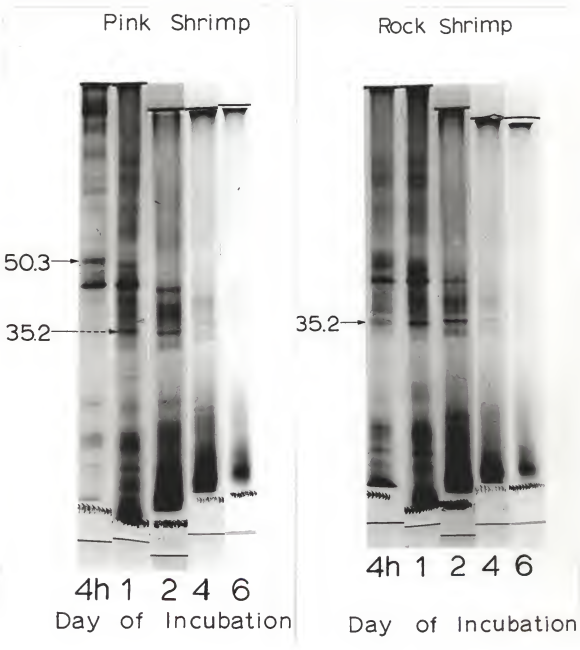


Figure 8. Serial changes in SDS-PAGE patterns of raw pink and rock shrimp extracted with 8 M urea. The numerical values are molecular weights of the specific proteins.

found. For white shrimp, identifiable bands in sections B and C were noticed for up to the seventh day after extraction (data not shown).

The smearing pattern for rock shrimp was prevented by shortening the protein incubation time in 8 M urea to 1 hr. The delay of the electrophoresis process to 4 hr introduced minor distortion of the pattern (Fig. 9). Dilution (about 20-fold) of the 8 M urea extract with water and/or electrophoresis buffer during sample preparation for electrophoresis prevented further changes in protein patterns even after storing at 4°C for 24 hr (Fig. 9). Thus, it was likely that the highly concentrated urea (8 M) used to extract shrimp proteins was responsible for the smeared patterns.

Conclusion

SDS-PAGE was an effective method to differentiate and identify species of raw or boiled shrimp. The water extract of each species of raw shrimp demonstrated a characteristic electrophoretic pattern. Heat-treatment of shrimp caused a loss of water-extractable proteins and no species-specific gel pattern was obtained. SDS greatly increased protein extractability and the number of proteins on the gel, thus making the identification of the genus of heat-treated shrimp possible. SDS-PAGE with the appropriate protein extraction system was found to be very effective in a blind study for identifying shrimp species. A maximum error of 10% was encountered to detect the actual amount of the individual shrimp in a rock-pink shrimp mixture.

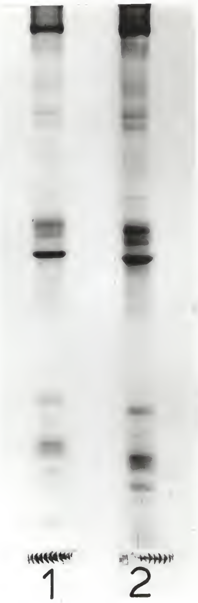


Figure 9. SDS-PAGE patterns of urea extracts of raw rock shrimp with (2) or without (1) dilution. Sample A was run within 1 hr after the SDS homogenate was prepared, and sample B was run after the sample was prepared, diluted to 35 μg/ 20 μL and stored at 4°C for 24 hr.

CHAPTER IV

SPECIES IDENTIFICATION OF RAW AND BOILED SHRIMP BY A UREA GEL ISOELECTRIC FOCUSING TECHNIQUE

Introduction

Isoelectric focusing (IEF) has been extensively used for seafood species identification because of its high resolution in separating proteins. IEF provides reliable and reproducible electrophoretic protein patterns for differentiating closely related species (Lundstrom, 1980).

Polyacrylamide and agarose are two common gel supports used for species identification by IEF. Ocean perch, monkfish, pollack, cod, wolffish, cusk, haddock and whiting have been identified with a 93% accuracy using thin layer polyacrylamide gel IEF (Lundstrom, 1980). The advantage of choosing agarose over polyacrylamide IEF is that it requires a shorter focusing time and it provides high resolution as well as reproducibility. Using thin layer agarose IEF, various fish species have been readily identified (Hamilton, 1982; Lundstrom, 1981b, 1983a).

Identification of cooked or processed seafood has not been extensively studied using this methodology. Protein molecules are readily altered by heat treatment. Urea has been widely used to reduce hydrophobic interactions between proteins (Cantor and Schimmel, 1980) and to increase their resolution in gel electrophoresis (O'Farrell, 1975). The genus of cooked or frozen crab meat was identified with 98% accuracy among 79 samples when crab meat proteins were extracted with urea and analyzed by IEF. This procedure, however, was not sensitive enough to identify individual species (Krzynowek and Wiggin, 1979, 1981). A similar result was shown by An et al. (1988) using an 8 M urea extract of boiled shrimp proteins for analysis with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The genus of the cooked shrimp was properly identified but not the species. The authors also used 1% SDS to extract boiled shrimp proteins and reported results comparable to the urea extracts. Urea has also been incorporated in electrophoretic gels to enhance protein separation and resolution (Boulikas, 1985; Keung et al., 1985; O'Farrell, 1975; Tuszynsky et al., 1978). By utilizing a 9.2 M urea gel in IEF for the first dimension, O'Farrell (1975) successfully separated an Escherichia coli lysate into over 1,000 components by two-dimensional gel electrophoresis.

The present study was carried out (1) to investigate if the addition of urea in IEF polyacrylamide gels would enhance the resolution of protein patterns for shrimp species identification, (2) to optimize urea IEF conditions for speciation of raw and cooked shrimp, and (3) to examine the species-specificity of shrimp protein banding patterns by combining the protein extraction systems with urea gel IEF.

Materials and Methods

Shrimp Samples

Three shrimp species, pink (*Penaeus duorarum*, Key West, FL), white (*Penaeus setiferus*, Jacksonville, FL) and rock shrimp (*Sicyonia brevirostris*, Port Canaveral, FL), were harvested and transported within 48 hr to the Food Science and Human Nutrition Department at the University of Florida. The samples were stored at -33°C until needed. Individual shrimp were thawed under tap water, peeled, and deveined. For the cooking treatment, shrimp were placed in boiling water for 5 min. The experimental protocol was repeated more than 10 times.

Protein Extraction and Sample Preparation for Gel Electrophoresis

Water, 1% SDS (w/v, 95% purity, Sigma Chemical Co., St. Louis, MO), and 8 M urea (electrophoresis purity, BioRad) were used to extract proteins from raw or cooked shrimp (An et al., 1988). All three solvents contained 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM EDTA and 0.01% (w/v) sodium azide to inhibit proteases and microbial growth. Both raw and cooked shrimp samples were chopped and each combined in separate beakers with the individual solvents at a ratio of 1:3 (w/v). Samples were homogenized at room temperature for 1 min using a Polytron, and then centrifuged for 20 min at 26,900 x *g* (20°C). The supernatants were collected and the protein concentration determined (Lowry et al., 1951); the final concentration was adjusted to 5 µg/µL with water. The presence of SDS (0.01%) or urea (0.08 M) in the diluted samples did not interfere with the Lowry protein assay. After 3-4 granules of sucrose were added to each 100 μL of extract, and 100 μg of protein was applied to the IEF gel.

For mixture samples, shrimp extracts were mixed in a 1:1 protein ratio (w/w) between pink and white, pink and rock, or white and rock and a 1:1:1 ratio between all three species. Raw shrimp mixtures were extracted only with water, while cooked shrimp mixtures were extracted with water as well as 1% SDS. The mixtures were processed as above and subjected to IEF analysis.

Optimization of IEF Conditions

The IEF method of O'Farrell (1975) was modified for a slab gel system. Various urea and ampholyte concentrations in the gel as well as focusing time were compared to establish the best conditions for shrimp species identification.

Urea concentration. A non-denaturing (without urea) and a denaturing gel system with 6 or 9.2 M urea were compared. The water extract from raw pink shrimp and protein standards (Broad pI kit, pH 3-10, Pharmacia, Piscataway, NJ) were analyzed using a polyacrylamide gel containing 6.2% (v/v) ampholyte (Pharmalyte pH 3-10, Pharmacia). The protein standard mixture contained: trypsinogen, pI 9.30; lentil lectin-basic band, pI 8.65, -middle band, pI 8.45, -acidic band, pI 8.15; horse myoglobin-basic band, pI 7.35, -acidic band, pI 6.85; human carbonic anhydrase B, pI 6.55; bovine carbonic anhydrase, pI 5.85; ß-lactoglobulin A, pI 5.20; soybean trypsin inhibitor, pI 4.55; and amyloglucosidase, pI 3.50. Proteins were focused at room temperature for 17 hr at 400 V with a circulating tap water coolant system.

Ampholyte concentration. Banding patterns of the water extract of raw pink shrimp and protein standards were compared using a 9.2 M urea polyacrylamide gel containing 5.0 or 6.2% (v/v) ampholyte. The gel was focused for 17 hr.

Focusing time. Two focusing times, 10 and 17 hr, were compared using a 9.2 M urea gel containing 6.2% (v/v) ampholyte.

Isoelectric Focusing

A gel mixture containing 4% (w/v) acrylamide, 2% (w/v) Triton X-100 and 9.2 M urea was heated to 37°C with shaking for five min to dissolve urea. Ampholyte at a final concentration of 6.2% (v/v) was added, the gel mixture was degassed for 5 min, then combined with fresh persulfate solution and TEMED (final concentration: 0.02% (v/v) and 0.14% (v/v), respectively) and poured into 16 x 20 cm slab gel plates (0.75 mm thick) assembled with a comb. The gel was allowed to polymerize for 1 hr. Lysis buffer containing 9.5 M urea, 2% (w/v) Triton X-100 and 2% (v/v) ampholyte was overlayed on the gel following the removal of the comb. The gel was allowed to sit for another hour. After the lysis buffer was replaced with a fresh one, prefocusing of the gel was done at 200 V for 15 min, 300 V for 30 min and then 400 V for 30 min in a Protean II electrophoresis slab gel unit using

10 mM phosphoric acid as the anode solution and 20 mM sodium hydroxide as the cathode solution.

Both the lysis buffer and cathode solution were removed after prefocusing. Following the application of protein samples to the gel, an aqueous solution containing 2% (w/v) Triton X-100 and 2% (v/v) ampholyte was overlayed atop the protein samples. The gel plate was reassembled in the electrophoresis unit and fresh cathode solution was added to the chamber. Proteins were focused at room temperature for 17 hrs at 400 V with circulating tap water. After the IEF run, the gel was stained with Coomassie blue R-250 and destained (Anonymous, 1986).

Determination of the Apparent pI Values of Shrimp Protein Bands

Apparent pI values of shrimp proteins were determined indirectly by comparing their R_f values on the gel with those of the protein standards. A pH profile of the whole gel was determined at room temperature using a micro-surface pH electrode (Ingold Electrodes Inc., Wilmington, MA). A linear relationship of gel pH and the R_f values of protein standards enabled determination of apparent pI values for the corresponding standards. The latter was used to determine the pI of individual shrimp proteins. From the apparent pI and R_f values of the protein standards and the R_f values of the shrimp proteins on the gel, the apparent pI values of the shrimp proteins were determined.

Results and Discussion

Optimization of IEF Gel Conditions

Urea concentration. The presence of urea in the gel greatly increased protein resolution. Among the three different urea concentrations used (0, 6 and 9.2 M), the 9.2 M gel provided the best protein banding pattern for raw pink shrimp and standards (Fig. 10). Proteins were well-focused at the anode side and the bands were highly resolved. Good protein separation also occurred with the 6 M urea gel, but the resolution was not as good as the 9.2 M gel. Four major bands were found with shrimp samples run on gels containing no urea. In addition, the banding patterns and the pI values of the protein standards were different in the gels with or without urea.

Ampholyte concentration. More stable protein banding patterns were obtained on gels containing 6.2% ampholyte (Fig. 11). Compared to the bands on the 5% ampholyte gel, protein bands on the 6.2% gel migrated less from the cathodic end where the sample was applied. Protein bands were more evenly distributed on the 6.2% ampholyte gel, while they were primarily focused near the anode side in the gel containing 5% ampholyte. In addition diffusion of protein bands occurred on the gel containing 5% ampholyte.

Focusing time. Focusing for 17 hr provided better band resolution than did a 10 hr period (Fig. 11). Proteins focused for only 10 hr were not completely resolved. This

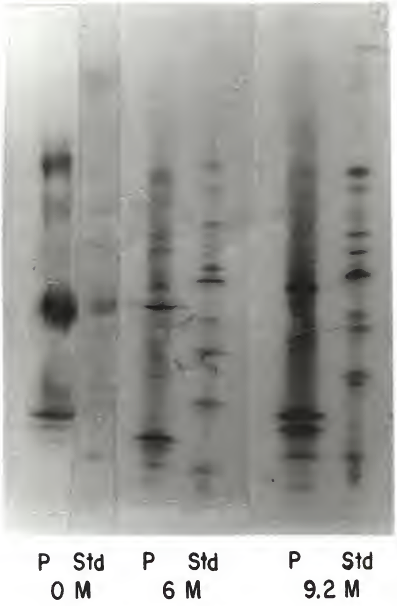


Figure 10. Protein banding patterns of the water extract of raw pink shrimp (P) and protein standards (Std) focused on polyacrylamide gel containing 0, 6, or 9.2 M urea (cathode on top). The numerical designations indicate apparent pI values of the protein bands.

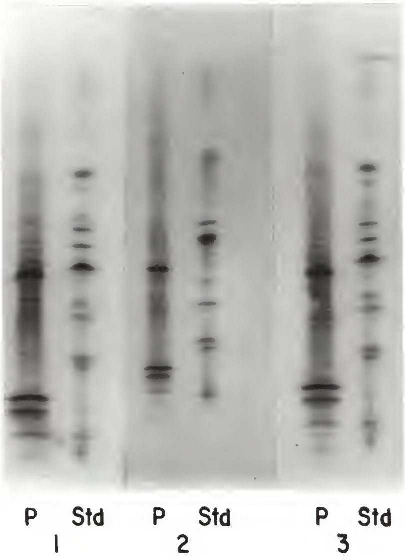


Figure 11. IEF patterns of the water extract of raw pink shrimp (P) and protein standards (Std) focused on polyacrylamide gel containing (1) 5% ampholyte for 17 hr, (2) 6.2% ampholyte for 10 hr, and (3) 6.2% ampholyte for 17 hr (cathode on top).

observation supported the results of Duncan and Hershey (1984) who showed that most proteins were completely localized by 6,400 volt-hour.

Determination of Apparent pI Values of Protein Bands

The pH gradient on the gel was linear over the entire length of the gel (r^2 >.99). The R_f values of protein standards on the 9.2 M urea gel were reproducible which was indicative of a stable pH profile. It has been reported that during electrofocusing the pI values of proteins in the presence of urea were different from the pI values without urea (Gelsema et al., 1979). However, we found that using the apparent pI value (determined by gel pH measurements at the band location) was useful to differentiate protein bands. Examples of the pI values of the protein standards and shrimp proteins determined from the standard curve of gel pH versus relative migration (R_f) were shown in figure 12.

Comparison of Solvent Extraction Systems

Water, SDS and urea extracts of raw pink shrimp had different overall IEF patterns (Fig. 12). The water extract showed banding patterns with high resolution. A new band with an apparent pI of 6.10 was found with the SDS extract. SDS usually caused a slight shift of the protein bands to higher apparent pI values. For example the band with an apparent pI of 6.75 with the water extract was found to have a pI of 6.86 with the SDS extract. This may be due to the binding of residual SDS to proteins which increases their negative charge.

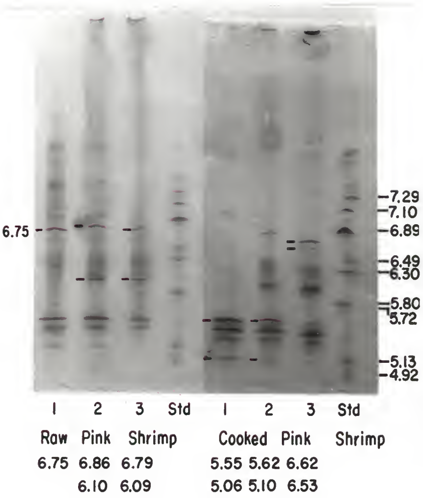


Figure 12. IEF patterns of raw and cooked pink shrimp extracted with (1) water, (2) 1% SDS, and (3) 8 M urea (cathode on top). Protein standards (Std) are also included. The numerical designations indicate apparent pI values of the protein bands.

SDS did not interfere with the focusing of proteins. Miller and Elgin (1974) showed that slight or no distortion of the pH gradient occurred when protein samples were applied in 1% or 0.1% SDS solution, respectively. The presence of urea might thus weaken SDS-protein interactions and cause the dissociation of SDS from proteins (Weber and Kuter, 1971; Miller and Elgin, 1974). Weber and Kuter (1971) showed that enzyme activity was regained after SDS was removed by an anion exchange column in the presence of 6 M urea.

Disappearance of protein bands occurred with the urea extract of raw pink shrimp. Bands with pI values of 6.79 and 6.09 were faded, possibly due to urea modification of the proteins.

Heat treatment of shrimp affected the extractability of proteins. Major pI bands of 5.06 - 5.55 resulting from water extracts of cooked pink shrimp (Fig. 12) were similar to those of the SDS extracts of cooked shrimp. The use of SDS or urea to extract cooked shrimp caused increases in the numbers of protein bands on the gel. Shrimp extracted by SDS or urea showed very similar banding patterns. The two bands with pI values of 6.62 and 6.53 were more distinct with urea than with the SDS extract. However, the five major pI bands of 5.10 - 5.62 that were useful for species differentiation of SDS extracts were distorted by urea extraction. Thus water and SDS were chosen to extract cooked shrimp proteins.

Species-specificity of Raw Shrimp Protein Patterns

IEF patterns of water extracts of raw pink, white and rock shrimp were compared. Water extracts gave rise to excellent banding patterns for species identification (Fig. 13). The most diverse and species-characteristic bands occurred at the low pI region. Bands with pI values of 5.48, 5.38, and 5.11 were specific for pink; 5.30, 5.22, and 4.97 for white; and 5.43, 5.20, and 5.06 for rock shrimp. Rock shrimp also had an additional specific band with a pI of 6.57. Numerous minor bands were also found in the water extracts but they were not chosen for species identification.

SDS extraction did not provide satisfactory results for species identification (data not shown). Although more bands were obtained with SDS extraction, the proteins were focused so close together that they could not be easily resolved to differentiate the shrimp species.

Species-Specificity of Cooked Shrimp Protein Patterns

Species differences with cooked shrimp were observed using SDS extracts (Fig. 14). The protein bands with low pI values were again most useful for species identification. Pink shrimp had characteristic pI bands of 5.57, 5.43 and 5.15; white shrimp--5.57, 5.41, 5.34 and 5.27; and rock shrimp--5.53, 5.32 and 5.12. Rock shrimp also had a unique band at 6.71.

The water extracts of cooked shrimp also showed species specificity (Fig. 15). Most of the protein bands found in water extracts of raw shrimp were not detected. The unique

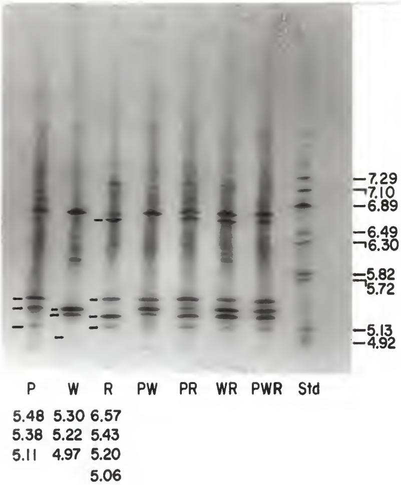


Figure 13. IEF patterns of water extracts of raw pink (P), white (W), and rock (R) shrimp, and their mixtures (cathode on top). Protein standards (Std) are also included. The numerical designations indicate apparent pI values of the protein bands.

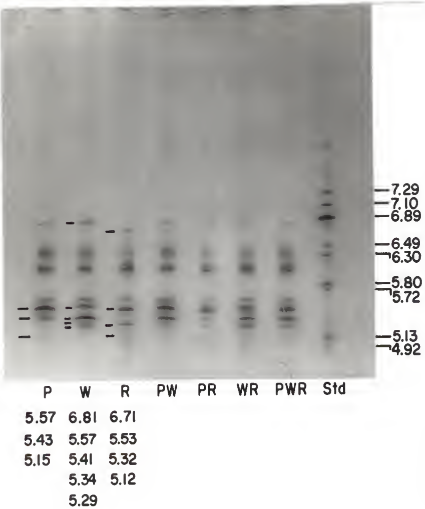


Figure 14. IEF patterns of SDS extracts of cooked pink (P), white (W), and rock (R) shrimp, and their mixtures (cathode on top). Protein standards (Std) are also included. The numerical designations indicate apparent pI values of the protein bands.

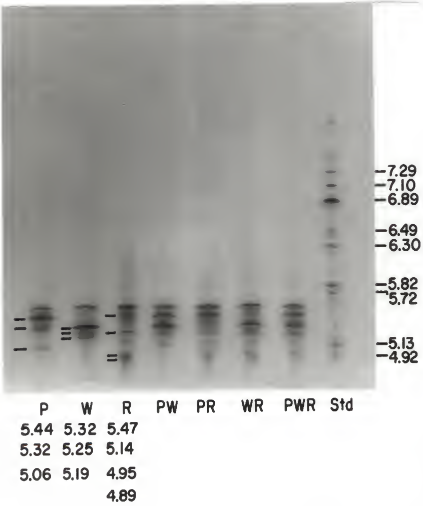


Figure 15. IEF patterns of water extracts of cooked pink (P), white (W), and rock (R) shrimp, and their mixtures (cathode on top). Protein standards (Std) are also included. The numerical designations indicate apparent pI values of the protein bands.

bands had low pI values: 5.44, 5.32 and 5.06 for pink; 5.32, 5.25 and 5.19 for white; and 5.47, 5.14, 4.95 and 4.89 for rock shrimp.

Comparison of Mixture Samples

The urea gel IEF system was used to detect the presence of shrimp species in various mixtures. The presence of rock shrimp could be easily detected in raw mixtures by the presence of a thick band at pI 6.57 (Fig. 13). The presence of pink or white shrimp in mixtures was distinguishable by the presence of bands with pI values of 5.11 and 4.97, respectively. The determination of shrimp origin in mixtures could possibly be determined from a comparison of the changes in intensity of the unique major pI bands of 5.20 - 5.48 in addition to the protein patterns. For example, pI bands of 5.48 and 5.38 appeared to be intensified in the pink-white shrimp mixture; pI bands of 5.43 and 5.20 appeared intensified for the pink-rock shrimp mixture; and pI bands of 5.30 and 5.22 appeared intensified for the white-rock shrimp mixture. When all three shrimp species were mixed, some overlapping of specific bands occurred and only three bands of similar intensity resulted. However, caution in using intensities should be observed because of the difficulty in reliable protein content, assay and measurement of the bands.

For boiled mixture samples, the presence of rock shrimp could easily be detected by the band at 6.71. The method used for raw samples was also applicable for cooked mixtures especially when samples were extracted with water. The detection of species could be made by comparing the changes of the intensity of the major bands with pI values of 5.12 - 5.57. The water extract (Fig. 14) provided a more distinguishable pattern than the SDS extract (Fig. 15) for mixtures of two or three shrimp species. The presence of pink shrimp in a mixture was detected by the appearance of a pI band at 5.06. The bands with pI values of 5.25 and 5.19, and 4.95 and 4.89 were unique for white shrimp and rock shrimp, respectively.

Thus, the urea gel IEF method described in this study was effective in identifying the species origin of raw and cooked shrimp. Water extracts provided excellent banding patterns to help differentiate raw pink, white and rock shrimp. The soluble proteins with low apparent pI values obtained from cooked shrimp by water and SDS also played important roles for shrimp species identification. This technique could be applied to identify the species of seafood or to detect adulteration in raw or heat-processed seafood products.

Conclusion

Incorporation of urea (9.2 M) in the gel greatly increased the protein separation and the band resolution. On the optimized condition using gels containing 9.2 M urea and 6.2% ampholytes and focused for 17 hrs, shrimp species were identified by the patterns of major protein bands. However, identification of species in the mixture samples were complicated due to the overlapping of protein bands for both raw and boiled shrimp.

CHAPTER V

USE OF A MODIFIED UREA GEL ISOELECTRIC FOCUSING TECHNIQUE FOR SPECIES IDENTIFICATION OF RAW AND BOILED SHRIMP

Introduction

Isoelectric focusing (IEF) has been extensively used for identification of seafood species because it provides reliable and reproducible resolved protein patterns for differentiating closely related species (Lundstrom, 1980). Using thin layer polyacrylamide gel IEF, various fish species have been readily identified (Hamilton, 1982; Lundstrom, 1981b, 1983a).

Identification of cooked or processed seafood has not been extensively studied using this methodology. Most difficulties encountered are due primarily to alterations of protein molecules by heat treatment. The extractability of heat denatured proteins can be increased by the use of sodium dodecyl sulfate (SDS) or urea. Krzynowek and Wiggin (1979, 1981) used IEF of urea-extracted crab meat proteins to identify with 98% accuracy the genus of cooked or frozen crab meat among 79 samples. Recently An et al. (1988) used 8 M urea or 1% SDS to extract boiled shrimp proteins, and they properly identified the genus of cooked shrimp using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Urea has been incorporated in electrophoretic gels to enhance protein separation and resolution (Boulikas, 1985; Keung et al., 1985; O'Farrell, 1975; Tuszynsky et al., 1978).

By utilizing a 9.2 M urea IEF gel for the first dimension, O'Farrell (1975) successfully separated an Escherichia coli lysate into over 1,000 components by two-dimensional gel electrophoresis. An et al. (1989a and b) also have achieved a good protein separation for shrimp, fish and surimi samples by adding 9.2 M urea and 6.2% ampholytes (pH 3-10) in the IEF gel. The results indicated that even cooked samples could be identified by this method. Although most of the major bands at the anodic side of the gel were resolved and could be used effectively for species comparison, the minor protein bands at the lower pI region of the gel were clustered together due to the pH 3-10 ampholytes (An et al., 1989a). Since these minor protein bands may also provide useful information to further aid species identification, efforts were made to expand that region by incorporating into the gel a narrower ampholyte range (pH 4-6.5) (Marshall and Williams, 1988). Therefore, the objectives of this study were (1) to optimize the propositions of pH 3-10 and pH 4-6.5 ampholytes to better separate species-specific protein bands at a narrower pH range from 5.0 to 7.5, and (2) to examine whether the IEF patterns could be used for species identification of the raw and cooked shrimp.

Materials and Methods

Sample Preparation

Three shrimp species, pink (Penaeus duorarum, Key West, FL), white (Penaeus setiferus, Jacksonville, FL), and rock shrimp (Sicyonia brevirostris, Port Canaveral, FL), were harvested and transported within 48 hr to the Food Science and Human Nutrition Department at the University of Florida. The samples were stored at -33°C until needed. Individual shrimp were thawed under tap water, peeled, and deveined. For the cooking treatment, shrimp were placed in boiling water for 5 min.

Water and 1% SDS (w/v) were used to extract proteins from raw or cooked shrimp, respectively (An et al., 1988). The solvents contained 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM EDTA and 0.01% (w/v) sodium azide to inhibit proteases and microbial growth. Both raw and cooked shrimp samples were chopped and each combined in separate beakers with the individual solvents at a ratio of 1:3 (w/v). Samples were homogenized at room temperature for 1 min using a Polytron, and then centrifuged for 20 min at 26,900 x g (20°C). The supernatants were collected and the protein concentration determined (Lowry et al., 1951); the final concentration was adjusted to 5 μg/μL with water. After 3-4 granules of sucrose were added to each 100 μL of extract, 100 μg of protein was applied on the IEF gel. For mixture samples, shrimp extracts were mixed in a 1:1 protein ratio (w/w) between pink and white, pink and rock, or white and rock and a 1:1:1 ratio between all three species. The mixtures were processed as above and subjected to IEF analysis. All experiments were repeated at least three times.

Optimization of a Narrower pH IEF Gel System

The modified IEF method of An et al. (1989a) for a slab gel system was further modified as follows.

Electrolytes. Two different electrolyte systems, 20 mM sodium hydroxide-10 mM phosphoric acid (O'Farrell, 1975) and 10 mM histidine-10 mM glutamic acid (Anonymous, 1988) were compared. Banding patterns were analyzed using the water extracts from raw pink shrimp and protein standards (Broad pI kit, pH 3-10, Pharmacia). The protein standards contained: trypsinogen, pI 9.30; lentil lectin-basic band, pI 8.65, -middle band, pI 8.45, -acidic band, pI 8.15; horse myoglobin-basic band, pI 7.35, -acidic band, pI 6.85; human carbonic anhydrase B, pI 6.55; bovine carbonic anhydrase, pI 5.85; ß-lactoglobulin A, pI 5.20; soybean trypsin inhibitor, pI 4.55; and amyloglucosidase, pI 3.50.

Ampholyte mixtures. Ampholytes were mixed to produce an optimal gel pH range for shrimp protein separation and to allow focusing at the anodic side of the pH 3-10 gel. Ampholyte 4-6.5 (Pharmalytes 4-6.5) at 100, 80, and 60% were mixed with 0, 20, and 40% ampholytes of pH 3-10 (Pharmalytes 3-10); the mixture was added into the urea gel at the final concentration of 6.2% (v/v). The banding patterns were compared with the water extract of raw pink shrimp and protein standards.

Isoelectric Focusing

A gel mixture containing 4% (w/v) acrylamide, 2% (w/v) Triton X-100 and 9.2 M urea was heated to 37°C with shaking for five min to dissolve urea. Ampholytes at a final concentration of 6.2% (v/v) were added, the gel mixture was degassed for 5 min, then combined with fresh persulfate solution and TEMED (final concentration: 0.02% (v/v) and 0.14% (v/v), respectively) and poured into 16 x 20 cm slab gel plates (0.75 mm thick) assembled with a comb. The gel was allowed to polymerize for 1 hr. Lysis buffer containing 9.5 M urea, 2% (w/v) Triton X-100 and 2% (v/v) ampholyte was overlayed on the gel following the removal of the comb. The gel was allowed to sit for another hour. After the lysis buffer was replaced with a fresh one, prefocusing of the gel was done at 200 V for 15 min, 300 V for 30 min and then 400 V for 30 min in a Protean II electrophoresis slab gel unit using 10 mM phosphoric acid as the anode electrolyte solution and 20 mM sodium hydroxide as the cathode solution.

Both the lysis buffer and cathode solution were removed after prefocusing. Following the application of protein samples to the gel, an aqueous solution containing 2% (w/v) Triton X-100 and 2% (v/v) ampholyte was overlayed atop the protein samples. The gel plate was reassembled in the electrophoresis unit and fresh cathode solution was added to the chamber. Proteins were focused at room temperature for 17 hrs at 400 V with circulating tap water. After the IEF run, the gel was stained with Coomassie blue R-250 and destained. Positive image was developed from the stained gel using EDP paper (Electrophoresis Duplicating Paper).

Protein profiles of the IEF gel was scanned using the video densitometer (Model 620, BioRad) with the developed pictures on EDP paper. The densitometer was set at the reflectance mode.

pH Measurement of the Gel and Determination of the Apparent pI Values of Shrimp Protein Bands

Apparent pI values of shrimp proteins were determined indirectly by comparing their R_f values on the gel with those of the protein standards. A pH profile of the whole gel was determined at room temperature ($25 \pm 2^{\circ}C$) before protein fixation using a micro-surface pH electrode. A linear relationship of gel pH and the R_f values of protein standards enabled determination of the apparent pI values for the corresponding standards. From the apparent pI and the R_f values of the protein standards and the R_f values of the shrimp proteins on the gel, the apparent pI values of shrimp proteins were determined.

Results and Discussion

Optimization of IEF Gel Conditions for a Narrower pH Range

Electrolytes. The histidine-glutamic acid electrolyte system recommended by the manufacturer interfered with proteins focusing at the narrower pH range of 4-6.5. There was no stable pH gradient formation in the gel and consequently no protein separation (data not shown). The interference still occurred when proteins were focused at $4^{\circ}C$

in the gels containing no urea. It was speculated that the low pH of histidine might cause protein precipitation on the gel and thus interfere with their entering into the gel. The precipitated proteins might also prevent electrolytes from moving continuously through the gel. This problem was alleviated by the use of the sodium hydroxide-phosphoric acid electrolyte system; a stable pH gradient and reproducible banding patterns were obtained. Therefore the sodium hydroxide-phosphoric acid system was used for subsequent studies.

Ampholyte mixtures. Different ampholyte mixtures affected the pH profile of the gel at the cathodic region. Incorporation of 40% pH 3-10 ampholytes with 60% pH 4-6.5 ampholytes caused the increase of about one pH unit at the end of the cathodic region as compared to the gel that contained only pH 4-6 ampholytes (Fig. 16). Thus the actual pH of the gel containing only pH 4-6.5 ampholytes was determined to range from 4.3 to 7.2; from pH 4.6 to 7.7 for the 80% pH 4-6.5 ampholytes; and from pH 4.3 to 8.3 for the 60% pH 4-6.5 ampholytes.

The change of the gel pH range also affected the resolution of raw pink shrimp proteins and protein standards. Band distance between proteins increased due to the higher percentages of the pH 4-6.5 ampholytes. Among the three different mixtures, 80% (pH 4-6.5 ampholytes) was found to separate efficiently the raw shrimp proteins. Most of the protein bands useful for shrimp species identification,

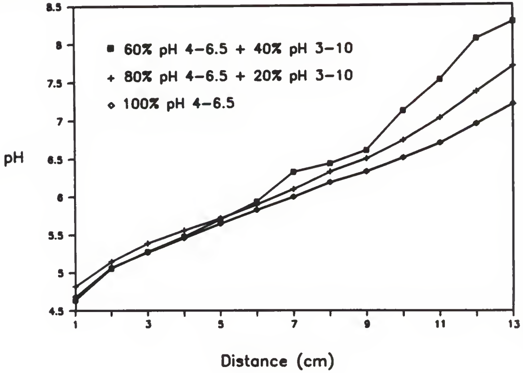


Figure 16. pH profiles of IEF gel containing three different ratios of wide and narrow pH range of ampholytes.

including those bands with low pI values reported previously (An et al., 1989a), were well resolved and distributed throughout the gel (Fig. 17). The use of only the narrow pH range ampholytes (4-6.5) showed a good protein band separation; but some protein bands at the cathodic end were excluded from the gel due to the narrower gel pH range. Although most of the protein bands found on the gel containing only pH 3-10 ampholytes were present on the gel containing the 40% (pH 3-10) and 60% (pH 4-6.5) ampholyte mixture, the protein bands were not separated clearly as those on the gel containing the 20% (pH 3-10) and 80% (pH 4-6.5) ampholyte mixture. Therefore the latter ampholyte mixture was used to separate shrimp proteins in further studies.

Species-Specificity of Raw Shrimp Protein Patterns

IEF patterns of the water extracts of raw pink, white and rock shrimp and their mixtures were compared to determine whether IEF patterns could be used to identify species (Fig. 18). As reported previously using only the pH 3-10 ampholytes (An et al., 1989a), species identification could be confirmed by detection of the major species-specific protein bands that were present at the anodic side (Fig. 18). These included the protein bands with pI values of 6.97, 6.79, 5.52 and 5.45 for pink shrimp, the 6.87, 5.45 and 5.42 bands for white shrimp, and the 5.54 and 5.31 bands for rock shrimp.

Species identification could also be achieved by comparing the patterns of the minor bands that were well separated. The bands with pI values of 7.41, 7.28 and 7.26 for pink shrimp,

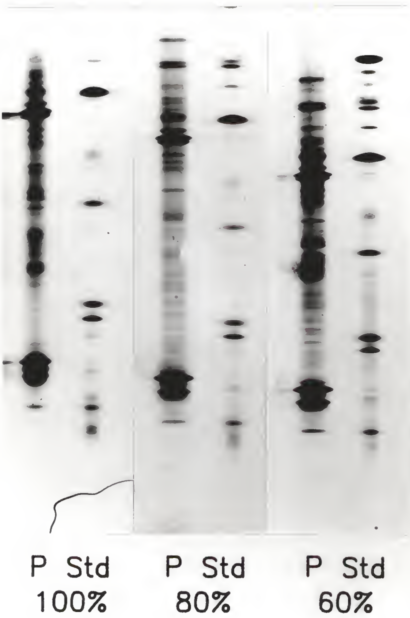


Figure 17. IEF patterns of water extracts of raw pink and protein standards focused on urea polyacrylamide gel containing 100%, 80%, and 60% of pH 4-6.5 ampholytes with addition of 0%, 20%, and 40% pH 3-10 (Cathode on top).

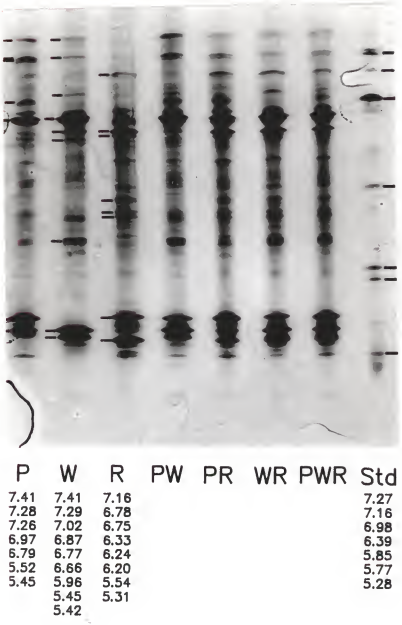


Figure 18. IEF patterns of water extracts of raw pink (P), white (W), and rock (R) shrimp, and their mixtures (cathode on top). Protein standards (Std) are also included. The numerical values indicate apparent pI values of the protein bands.

the 7.41, 7.29, 7.02, 6.77, 6.66 and 5.96 bands for white shrimp, as well as the 7.16, 6.78, 6.75, 6.33, 6.24 and 6.20 bands for rock shrimp were shown to be specific for each species. The presence of these bands together with the major bands could be used effectively in identifying the species as well as in detecting the presence of a specific shrimp species in the mixture. For example, the presence of rock shrimp in the pink + rock, white + rock, or pink + white + rock shrimp mixture could be determined from the presence of the 7.16, 6.75, 6.33, 6.24 and 5.31 protein bands that were specific for rock shrimp.

The difference in protein banding patterns of the three shrimp species was well reflected by the diversity of their densitometric scanning profiles (Fig. 19). Since better protein separation occurred in these gels containing the 20% (pH 3-10) and 80% (pH 4-6.5) ampholyte mixture, species-specific peaks were distributed throughout the profiles. Peaks at the 5-40 and 90-110 mm regions appeared to be more useful for species differentiation.

Species-Specificity of Cooked Shrimp Protein Patterns

The water extracts of the cooked shrimp also showed different protein patterns (Fig. 20). Less shrimp proteins were extracted due to heat treatment. Thus many minor protein bands that were used for species identification in the raw sample were not present on the gel. The bands with low pI values, such as the 5.56, 5.51 and 5.48 for pink shrimp, the 5.48, 5.43, 5.38, 5.07 and 4.98 for white shrimp, and the

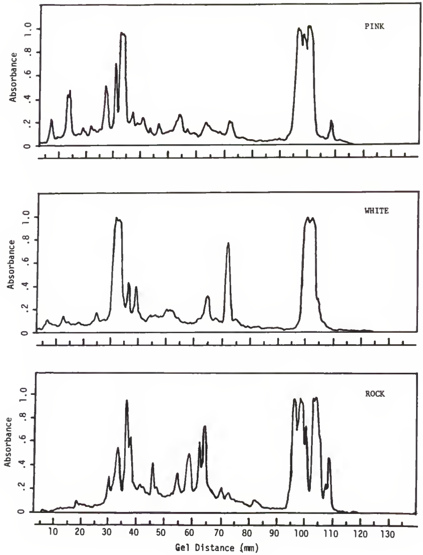


Figure 19. Densitometric profiles of water extracts of pink, white, and rock shrimp extracted with water.

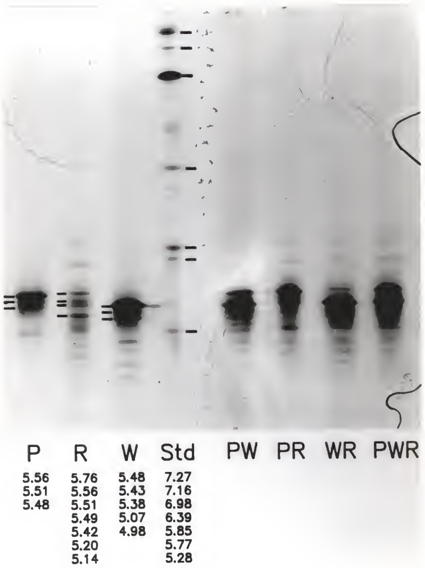


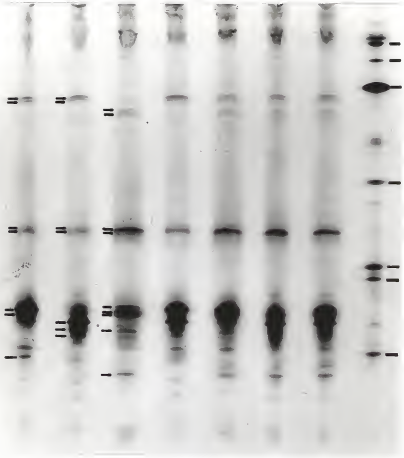
Figure 20. IEF patterns of water extracts of cooked pink (P), white (W), and rock (R) shrimp, and their mixtures (cathode on top). Protein standards (Std) are also included. The numerical values indicate apparent pI values of the protein bands.

5.76, 5.56, 5.51, 5.49 5.42, 5.20 and 5.14 for rock shrimp, were most useful for species identification. Identification of shrimp species in a mixture of shrimp, however, was complicated because of the similar patterns between the shrimp species.

SDS enhanced protein extractability of some proteins and thus caused the increase of protein band numbers on the IEF gel (Fig. 21). The protein bands with pI values of 6.83, 6.79, 6.03 and 5.13 were specific for rock shrimp; these rock shrimp bands could easily be distinguished even in mixture samples. Species could also be identified by checking the presence of the major protein bands with pI values of 5.33 and 5.26 for pink shrimp, and 5.40 and 5.23 for white shrimp. Due to the clustering of these bands, the identification of species specificity in mixture samples was difficult except for rock shrimp.

Conclusion

The use of a 20% (pH 3-10) and 80% (pH 4-6.5) ampholyte mixture in IEF gels caused the production of a narrower pH gel ranging from pH 4.7 to 7.7, and greatly enhanced protein separation and band resolution as compared to gels containing only the pH 3-10 ampholyte. In addition to the major bands, many species-specific minor bands were well separated in the water extracts of raw shrimp samples, thus providing important information for species identification. For cooked samples, each species could be differentiated by its own unique protein banding patterns. However, the differentiation of the



P	W	R	PW	PR	WR	PWR	Std
6.93	6.87	6.83					7.27
6.90	6.91	6.79					7.16
6.10	6.08	6.06					6.98
6.07	6.06	6.03					6.39
5.57	5.49	5.57					5.85
5.54	5.43	5.53					5.77
5.49	5.40	5.51					5.28
5.33	5.23	5.42					
5.26		5.13					

Figure 21. IEF patterns of SDS extracts of cooked pink (P), white (W), and rock (R) shrimp, and their mixtures (cathode on top). Protein standards (Std) are also included. The numerical values indicate apparent pI values of the protein bands.

specific shrimp species in a mixture of shrimp was not effective due to the loss of some species-related protein bands by the heat treatment. Unlike the pink and white shrimp, the presence of cooked rock shrimp in mixtures could be easily identified by using the unique protein bands that were present in the 1% SDS extractant of the cooked rock shrimp.

CHAPTER VI
IMMUNOCHEMICAL IDENTIFICATION OF SHRIMP SPECIES USING ROCK SHRIMP AS A MODEL

Introduction

The establishment of reliable procedures to identify food components has been of great research interest and commercial importance. Immunological techniques, such as immunodiffusion, hemagglutination inhibition, rocket immunoelectrophoresis, and enzyme-linked immunosorbent assay (ELISA) have been widely used for detecting the species origin of fish, beef, pork, and chicken or to detect food components in seafood or meat products (Hayden, 1977, 1978 and 1981; Hitchcock et al., 1981; Karpas et al., 1970; Skerritt, 1985). Among these methods, ELISA offers great advantages in that (1) the test can be performed in a short period of time, (2) a small volume of species-specific antisera is required, (3) the antisera can be mixed for multiple species specificity in a screening procedure, (4) equipment is available to semi-automate the assay, and (5) the increased sensitivity of the assay allows the use of simple sampling techniques (Whittaker et al., 1983). However, in early studies, the ELISA technique could not reliably differentiate the species of origin of meat samples because of the cross-reactivity of conventional antisera with unrelated species, the variable titer, and specificity of different preparations of antisera

(Kang'ethe et al., 1982; Patterson et al., 1984; Whittaker et al., 1983). These problems with conventional antisera can be alleviated by the use of monoclonal antibodies (McAb) specific for different food components.

The hybridoma technique first introduced by Kohler and Milstein (1975) is used to produce unlimited quantities of McAbs with exceptional specificity and consistent binding properties (Edwards, 1981). Therefore McAbs should be very effective reagents in ELISA tests developed to identify the species origin of food components as well as to quantitate component levels in meat or seafood products. Furthermore, ELISA tests using McAbs should be able to replace the less sensitive and more time-consuming electrophoretic methods which have been used officially for identifying fish species (AOAC, 1984).

Hybridoma technology was applied by Lundstrom (1984, 1985) to identify fish species and to differentiate fish stock. Crude protein extracts from heat-denatured fish muscle were used to immunize mice for the preparation of McAbs. However, high cross-reactivity between unrelated fish was noted, and species-specificity was not found when McAbs were tested against numerous fish species. This may have been due to the fact that most of the antibodies produced against the crude extracts were specific for the most prevalent proteins such as albumin rather than the less abundant species-specific proteins in the extracts. Similar results were also reported by Tracy et al. (1983) who injected a protein extract of whole human urine into mice in an attempt to produce McAbs of various specificities. Most of the positive hybridomas produced antibodies to albumin, an abundant urinary protein in renal disease, rather than the protein of interest, PC-30. After the antigens were separated by two-dimensional gel electrophoresis, McAbs were developed and shown to be specific for PC-30 as confirmed by autoradiography.

The present study was carried out to increase the specificity of immunochemical assay systems for species identification by using isolated component proteins as antigens to develop species-specific McAbs for fast screening of seafood species. The rock shrimp (Sicyonia brevirostris) was used as a model in order to determine the feasibility of this approach.

Materials and Methods

Preparation of Water-soluble Proteins

Water-soluble proteins were extracted from seafood and meat sources as previously reported (An et al., 1988) and analyzed for protein content (Lowry et al., 1951).

Purification of Protein "C"

Rock shrimp protein bands with MW of 17.7 and 18.5 kD, referred to as protein "C", were shown in a report (An et al., 1988) to be specific for rock shrimp when compared with pink or white shrimp using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein "C" was eluted from the 12% SDS-PAGE slab gels of the water extract of rock shrimp proteins. The gels were homogenized in water for 1 min using a Polytron (Brinkmann Instruments, Westbury, NY), and the mixtures centrifuged at 2000 x g for 20 min at room temperature using a bench-top IEC clinical centrifuge (International Equipment Co.). Eluted proteins in the supernatant were pooled and dialyzed overnight at 4°C in a membrane tubing (molecular weight cutoff of 6-8 kD, Spectrum Medical Industries, Inc., Los Angeles, CA) against an excess amount (about 100X in volume) of phosphate buffered saline (PBS, pH 7.4) containing 1.4 M NaCl, 0.1 M Na_2HPO_4 and 0.3 M KH_2PO_4, with constant stirring to remove residual SDS. The dialyzed protein "C" was checked for protein content and purity using SDS-PAGE following previously described procedures (An et al., 1988).

Two-dimensional Gel Electrophoresis of Rock Shrimp Water Extract

First and second dimensional gels of urea IEF and SDS-PAGE, respectively, were prepared and electrophoresed as previously described in the "Materials and Methods" section of the chapters III and IV with a slight modifications. The first dimensional electrophoresis was done with a tube gel (11.5 cm x 1.7 mm). Rock shrimp protein extract at 150 μg was applied, and the gel was focused in the Pharmacia Electrophoresis Apparatus GE-4II unit. After the focused gel was taken out of the tubes, equilibrated in the Tris-HCl buffer (pH 6.8) for 1 hr, the gel was placed on the top of the SDS-PAGE slab gel consisting of the stacking gel (3% total acrylamide) and the separating gel (12% total acrylamide) and maintained in position with 1% agarose. The slab gel was electrophoresed until bromophenol blue reached the bottom of the gel. The proteins were stained with Coomassie blue R-250 and destained as described in the chapter III.

Immunization

Four 6-week-old Balb/cBYJ female mice (Jackson Labs, Bar Harbor, ME) were each immunized with 100 μg of protein "C" freshly emulsified in RIBI's adjuvant (Monophosphoryl Lipid A-Trehalose Dimycolate, RIBI Immunochem Research, Inc., Hamilton, MT). The mixture was injected subcutaneously in 50 μL aliquots into each of four separate ventral sites, as well as in 150 μL amounts intraperitoneally and into one anterior dorsal subcutaneous site. The injection process was repeated three times at two-week intervals and the titer of the serum against protein "C" was determined by ELISA, as described below, one week after the second and third booster injections. A final boost was given 26 days after the third booster and four days prior to the fusion.

Development of Monoclonal Anti-protein "C" Antibodies

Monoclonal antibodies against protein "C" were produced using previously established protocols of Kao and Klein (1986). Spleen cells harvested from immunized mice were fused with SP2/0 myeloma cells at a ratio of 7.5:1 (spleen cells: myeloma cells) using 50% polyethylene glycol 1540. The fused cells were suspended in hypoxanthine-aminopterin-thymidine (HAT) selective medium and seeded into 96-well flat-bottom culture plates. Ten to 14 days later, the supernatants of growth-positive wells, as checked under an inverted microscope, were screened for reactivity of protein "C" and rock shrimp protein extracts by ELISA and immunodot blotting. Hybridomas producing anti-protein "C" antibodies were cloned by the limiting dilution method (Zola and Brooks, 1982). McAb isotypes were determined with ScreenType™ (Boehringer Mannheim Biochemicals, Indianapolis, IN) as described below.

Enzyme-linked Immunosorbent Assay for Protein "C"

A 96-well Nunc immunoplate (Denmark) was coated overnight at 4°C with 100 μL of 5 μg/mL protein "C" or water protein extracts in PBS containing 0.02% azide, pH 7.4 (PBS-az). The plate was washed three times with PBS containing 0.5% Tween-20 and 0.02% azide (PBS-Tw). The unbound sites were then blocked with 1% bovine serum albumin (BSA) in PBS for 1 hr at 4°C and incubated at room temperature for 1 hr with supernatants of hybridoma cultures or purified McAb, followed by plate washing three times with PBS-Tw. The plates were subsequently incubated for 1 hr with the rabbit anti-mouse IgG conjugated with alkaline phosphatase (Sigma Chem. Co., St. Louis, MO), followed by plate washing with PBS-Tw. Positive reactivity was detected by color development after the addition of the substrate, p-nitrophenylphosphate (Sigma) and incubation of the plate for 45 min in the dark. Absorbance was read at 405 nm using an ELISA reader (Molecular Devices, Palo Alto, CA).

Immunodot Blotting

Protein extracts, 20 μg in 20 μL, were dotted on nitrocellulose membrane paper (BA85, Schleicher and Schuell, Keene, NH) assembled in a Minifold I dot blot apparatus with incubation plate (Schleicher and Schuell) and incubated for 1 hr. Unbound sites on the membrane paper were blocked by incubating the paper in a PBS-az containing 5% nonfat milk for 1 hr. Following that, the membrane was incubated in the purified McAb (100 μg/mL) in 1% BSA in PBS-az for 30 min. After rinsing for 5 min in PBS-az containing 0.2% Tween-20 (PBS-T) and incubating with 1 μg/mL rabbit anti-mouse IgG conjugated with alkaline phosphatase for 30 min, the membrane was washed again in PBS-T and incubated in 0.1 mg/mL nitroblue tetrazolium and 0.05 mg/mL 5-bromo-4-chloro-3-indolyl phosphate in 0.1 M Tris buffer containing 1 mM $MgCl_2$ (pH 8.8) until color development. Positive samples containing protein "C" or rock shrimp were identified by the development of a blue color at the site of antigen immobilization.

Western Blotting

Crude protein extracts of pink, white, and rock shrimp were electrophoresed on 12% SDS-PAGE gels (An et al., 1988) and blotted onto Immobilon membrane paper (Millipore Corp., Bedford, MA) at 4°C using a Trans-Blot device (Bio Rad, Richmond, CA) at 50 mA for 2 hours. The proteins transferred onto the membrane could be visualized by the same techniques which were used for immunodot blotting with only slight modifications as follows. The membrane paper was blocked in the 5% nonfat milk in PBS-az for 2 hr, followed by 2 and 1 hr incubations, respectively, in solutions containing the purified McAb and the alkaline phosphatase conjugate.

Isotyping of Antibodies

Antibody isotypes were determined using the Screen Type™-Enzyme Immunoassay Kit for Screening/Isotyping of Murine Monoclonal Antibodies (Boehringer Mannheim Biochemicals, Indianapolis, IN). ELISA plates coated overnight at 4°C with protein "C" from rock shrimp were blocked with 1% BSA in PBS for 1 hr at room temperature. Culture supernatants to be determined for isotyping were added to individual sets of wells and incubated for 30 min at 37°C. To different sets of primary antibody-coated wells, class- or isotype-specific goat antibodies (anti-mouse IgG_1, anti-mouse IgG_{2a}, anti-mouse IgG_{2b}, anti-mouse Ig_{G3}, anti-mouse IgM, anti-mouse kappa, anti-mouse lambda) were added and incubated for 30 min at 37°C. Following the addition of peroxidase-conjugated swine anti-goat IgG to each well and the plates incubated for 30 min at 37°C, both ABTSR chromogen and the substrate were added. The plates were incubated at room temperature until a yellow color was developed. The absorbance of each well was read at 405 nm using an ELISA reader. The class or isotype of each McAb was determined from the wells that had absorbance values at least 2-3 times as high as those of negative controls.

Production of Ascites

Three to four male Balb/cBYJ mice were primed by intraperitoneal (i.p.) injection of 0.5 mL of pristane (2,6,10,14-tetramethylpentadecane, Sigma) one week prior to the i.p. injection of $4.7\text{-}5.0 \times 10^6$ cloned hybridoma cells in 0.5 mL of PBS. Ascites was collected by tapping the peritoneal cavity using a 20 gauge needle. The collection process started one week to ten days after injection of hybridoma cells and was repeated every few days until the animals died. The pooled ascites fluid was centrifuged at 2000 rpm for 15 min to remove the cells. The supernatant was frozen and stored until needed.

Purification of Monoclonal Antibodies

Monoclonal antibody (IgG_1 class) was purified from mouse ascites fluid by affinity chromatography using protein-A Sepharose 4B (Sigma) according to the methods of Ey et al. (1978) with some modifications as described below. Frozen ascites fluid was thawed and centrifuged at room temperature at 8,000 rpm in a microcentrifuge for 5 min. The supernatants were collected and diluted ten-fold with a buffer containing 1.65 M glycine and 3.3 M NaCl at pH 8.9, and filtered through a 0.45 μm filter (Gelman Sci., Inc., Ann Arbor, MI). The filtered ascites was loaded on a protein-A Sepharose column pre-equilibrated with a binding buffer (1.5 M glycine and 3 M NaCl at pH 8.9). The column was washed with the binding buffer to remove unbound proteins, and then the IgG_1-McAb was eluted with 100 mM citric acid at pH 6.0 and collected in fractions of approximately 1.0 mL each. The absorbance of the fractions was measured at 280 nm, and the fractions containing the McAb were pooled together. The column was regenerated by eluting all proteins remaining bound with 100 mM citric acid at pH 3.0. The purified McAb showed two distinct bands (light and heavy chains) by SDS-PAGE on the 12% polyacrylamide gels in the presence of ß-mercapthoethanol.

Pretreatment of antigens. Crude water extracts of rock, pink, and white shrimp were divided into native and heat-treated groups. For the heat-treated group, the water extract was boiled for 5 min in water and centrifuged at 8,000 rpm for 5 min to remove protein aggregates. Both the native and heat-treated groups were analyzed for protein content using the Lowry method, diluted to 5 μg/mL, and then added with or without SDS (final concentration of SDS, 0.1%, w/v). The samples were each reacted with McAb (4H2-10D3) at 100 μg/mL on ELISA and immunodot blot to determine if such treatments would increase the reactivity of rock shrimp proteins with this McAb.

Optimization of McAb and Antigen Concentrations for ELISA

Antibody concentration. Concentrations of McAb (4H2-10D3) at 200, 100, 50, 10, 5, 1, 0.5 μg protein/mL were tested by ELISA to determine the optimal concentration needed to achieve highly specific reactivity for rock shrimp proteins. Only the heat-treated and diluted water extracts of rock, pink or white shrimp at 5 μg protein/mL were used to coat the ELISA plates.

Antigen concentration. The heat-treated and processed water extracts of pink, white and rock shrimp at 500, 100, 50, 10, 5, 1, or 0.5 μg protein/mL were coated in a volume of 0.1 mL in each well of the ELISA plate. 4H2-10D3 McAb at 100 or 50 μg protein/mL was tested on these plates to determine the optimal concentration of antigen needed for maximum reactivity in the ELISA assay.

Blind Study to Detect and Quantitate Rock Shrimp

Blind studies were carried out to confirm the specificity of the 4H2-10D3 McAb for rock shrimp and to determine the ability of the ELISA assay to detect rock shrimp present in a panel of food samples.

Blind study I. A single blind test using ELISA was employed to determine the specificity of the 4H2-10D3 McAb for rock shrimp protein. The reactivity of rock shrimp was tested along with 23 other species of seafoods including oyster, scallop, lobster, clam, fishes and shrimp obtained from various locations, and meats including chicken, pork and beef. Water-soluble proteins of these diverse food samples were extracted following the same procedures used to extract rock shrimp proteins (An et al., 1988). The extracts were heat-treated for 5 min in boiling water followed by centrifugation at 8,000 rpm for 5 min. After the protein content of each extract was determined by the Lowry method, the protein concentration of each sample was adjusted to 10 μg/mL and a 100 μL aliquot was analyzed by ELISA. Experiments were performed in two replications.

Blind study II. The sensitivity of the McAb in detecting rock shrimp in sample mixtures containing seafood and meat samples was conducted in a double-blind study using the ELISA test. The water extracts of the 26 seafood and meat samples, after heat treatment and centrifugation, were each adjusted to a protein content of 500 μg/mL. They were combined randomly with each other or with PBS in various ratios unknown to the author to yield 24 sample mixtures (Table 4). The sample mixtures were diluted to 10 μg protein/mL, and aliquots of 100 μL of each mixture were analyzed by ELISA with 100 μg/mL McAb. This experiment was repeated once.

Those mixtures that were shown from the ELISA test to contain rock shrimp, (determined after revealing the blind listing of the sample mixtures), were then serially diluted with PBS (1:5, v/v) to 5^{-7} of the original protein content. Each of the serially diluted samples was then subjected to the above mentioned ELISA test to determine the lowest amount of rock shrimp protein that could be detected using the McAb. The most diluted samples which gave O.D. values greater than 0.2 were marked. The experiment was repeated once. The rock shrimp protein content of these diluted samples were calculated using the following equation (refer to Table 9):

Rock shrimp content (ng) =
(Protein content, ng) x (Rock shrimp percentage) x
(Dilution factor)

Results and Discussion

SDS-PAGE Banding Patterns of Various Seafood and Meat Samples

Seafood species and meat samples each showed a distinct SDS-PAGE protein pattern useful for species identification (Fig. 22). The protein profile of rock shrimp was significantly different from those of the other seafoods and meats including clams, oysters, sea scallops, fish, beef,

Table 4. Composition of various unknown mixture samples used in Blind Study II.

Sample No.	Percent composition (v/v) of various seafood and meat protein extracts
Z1	50% TR + 50% SS
Z2	50% S2 + 50% B
Z3	50% S + 50% F
Z4	50% R2 + 50% PBS
Z5	50% R1 + 50% PBS
Z6	50% S7 + 50% S9
Z7	50% O + 50% TU
Z8	50% S10 + 50% L1
Z9	50% S5 + 50% C
Z10	50% G + 50% P
Z11	50% S3 + 50% L2
Z12	50% S8 + 50% S4
Z13	50% R3 + 50% PBS
Z14	33% CH + 33% S1 + 33% S6
Z15	33% R2 + 33% O + 33% SS
Z16	33% R2 + 33% C + 33% S10
Z17	33% R3 + 33% S4 + 33% S1
Z18	33% R3 + 33% CH + 33% L2
Z19	33% R1 + 33% S10 + 33% L1
Z20	17% R1 + 17% R2 + 17% R3 + 33% G + 17% PBS
Z21	17% TR + 17% SS + 17% S2 + 17% B + 33% R2
Z22	17% TR + 17% S + 17% F + 17% R1 +17% S7 + 17% S9
Z23	17% O + 17% SS + 17% C + 17% S10 + 33% R2
Z24	29% R1 + 14% S5 + 29% P + 29% PBS

Abbreviations designate: R1, rock shrimp from Port Canavarel, FL; R2, rock shrimp from Cedar Key, FL (February); R3, rock shrimp from Cear Key, FL (December); S1, white shrimp from Ecuador; S2, Argentine-red shrimp from Argentine; S3, Blue shrimp from Ecuador; S4, pink shrimp from Tampa, FL; S5, white shrimp from Peru; S6, brown shrimp from North Carolina; S7, white shrimp from Columbia; S8, white shrimp from Georgia; S9, white shrimp from Honduras; S10, blue shrimp from Honduras; L1, lobster from Australia; L2, lobster from Florida; C, clam; O, oyster; SS, sea scallop; TR, trout; S, salmon; F, flounder; TU, tuna; G, grouper; B, beef; P, pork; CH, chicken.

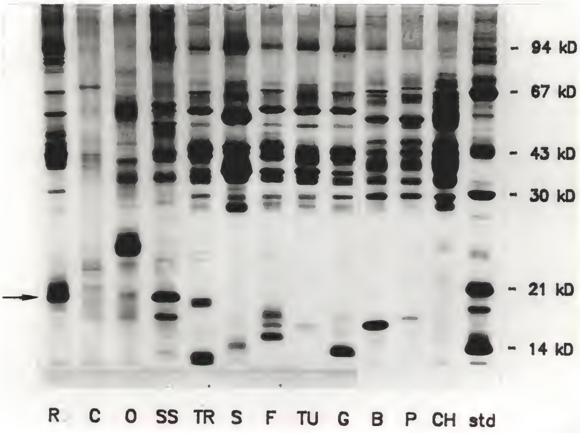


Figure 22. SDS-PAGE profiles of water-soluble proteins from various seafood and meat (cathode on top). The arrow designates protein "C". R, rock shrimp from Cear Key, FL; C, clam; O, oyster; SS, sea scallop; TR, trout; S, salmon; F, flounder; TU, tuna; G, grouper; B, beef; P, pork; CH, chicken; std, low molecular weight protein standards with the individual molecular weight marked on the side.

pork, and chicken. The most significant variations of the profiles among the samples occurred in the lower one-third section of the gel (in the anodic region). Protein bands (with MW <25 kD) shown in this section were unique for each sample in terms of the number of bands and their molecular weights (as estimated by the relative migration, R_f). The two major bands unique for rock shrimp found in this section were referred to protein "C".

The protein profiles of various shrimp samples and lobsters from various parts of the world were also compared with those of rock shrimp (Fig. 23). In general, the profiles for pink, white, blue and brown shrimp were relatively similar. An et al. (1988) also reported that the SDS-PAGE protein profiles of the water extracts of pink and white shrimp were very similar. The protein pattern of lobster was different from that of rock shrimp as well as those of the other 10 shrimp samples. The movement of the two major bands found in the lower one-third section of the gel for the 10 different pink, white, blue and brown shrimp samples were almost identical. When compared to rock shrimp protein "C", these two bands had slightly lower R_f values and thus higher molecular weights.

The protein "C" of rock shrimp was found to consist of more than two proteins as analyzed by two-dimensional gel electrophoresis (Fig. 24). It comprised 19.7% of the total water-soluble proteins as estimated from the intensity of the protein bands shown on the densitogram (Fig. 25 and Table 5).

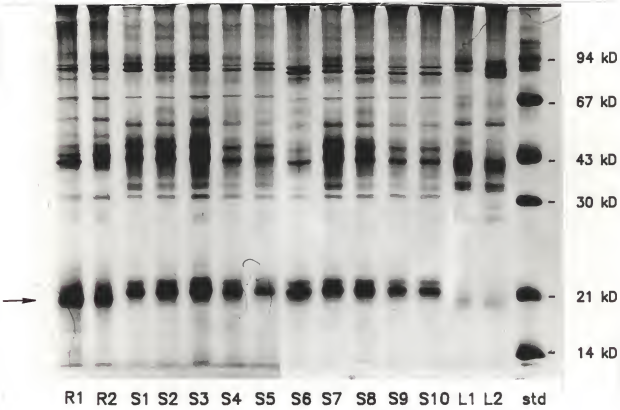


Figure 23. SDS-PAGE profiles of water-soluble proteins from various shrimp and lobsters (cathode on top). R1, rock shrimp from Port Canavarel, FL; R2, rock shrimp from Cedar Key, FL; S1, white shrimp from Ecuador; S2, Argentine-red shrimp from Argentine; S3, blue shrimp from Ecuador; S4, pink shrimp from Tampa, FL; S5, white shrimp from Peru; S6, brown shrimp from North Carolina; S7, white shrimp from Columbia; S8, white shrimp from Georgia; S9, white shrimp from Honduras; S10, blue shrimp from Honduras; L1, lobster from Australia; L2, lobster from Florida; std, low molecular weight protein standards with the individual molecular weight marked on the side. Rock shrimp protein "C" also marked with an arrow.

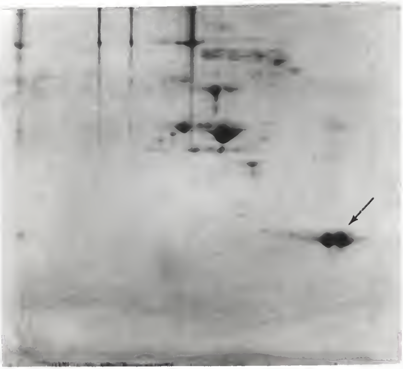


Figure 24. Two dimensional gel electrophoresis pattern of rock shrimp extracted with water. Protein "C" is marked with an arrow.

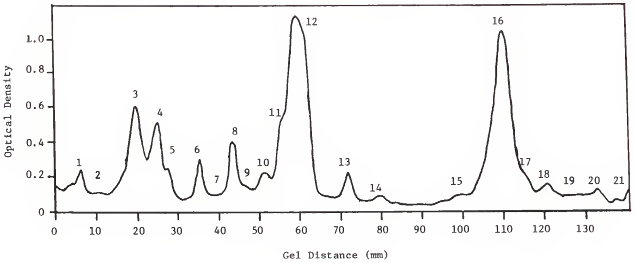


Figure 25. Densitometric scanning profile of the water-soluble rock shrimp proteins analyzed on a SDS-PAGE gel. Peak No. 16 is identified to be protein "C".

Table 5. The relative mobility and percent area of the various rock shrimp protein bands on the SDS-PAGE gel as determined by a gel scanner.

Peak No.	Relative mobility (R_f)	% Area
1	0.03	4.15
2	0.09	3.18
3	0.14	8.37
4	0.18	5.73
5	0.21	2.81
6	0.25	2.25
7	0.28	1.46
8	0.31	3.45
9	0.34	1.86
10	0.37	2.16
11	0.42	18.09
12	0.44	9.13
13	0.52	2.52
14	0.57	1.84
15	0.69	3.94
16	0.78	19.73
17	0.82	2.35
18	0.86	2.29
19	0.91	2.06
20	0.95	1.36
21	0.99	1.12

Protein "C" was isolated and eluted from the SDS-PAGE gel and used as a rock shrimp-specific antigen for immunization (Fig. 26).

Screening of Monoclonal Antibodies

Hybridoma cells derived from the fusion of antibody producing lymphocytes with myeloma cells were screened for their reactivity with protein "C" and rock shrimp extract. Out of the 400 wells seeded after the fusion, 330 were found to be hybridoma growth-positive. On initial screen by ELISA for reactivity with protein "C", 33 cell cultures were found to be protein "C"-positive (with O.D. reading > 0.4). They were transferred to and grown in 24-well tissue culture plates and the culture supernatants tested for rock shrimp specificity using pink and white shrimp extracts as negative controls. Eight of these 33 original cultures were shown by ELISA to have 3 to 10 times higher reactivity for rock shrimp than for pink or white shrimp (Table 6). A few negative cultures were found among the protein "C"-positive cultures that showed a high reactivity for pink or white shrimp but only negligible reactivity for rock shrimp. These negative cultures were not studied any further.

The reactivity of the antibody produced by these eight cultures was further tested by immunodot blot; all of them failed to react with the water extracts of pink and white shrimp (Fig. 27). The reaction with the rock shrimp extract only produced a very faint blue color. However, three cultures, 2E8, 4H1 and 4H2, showed positive reactions for rock

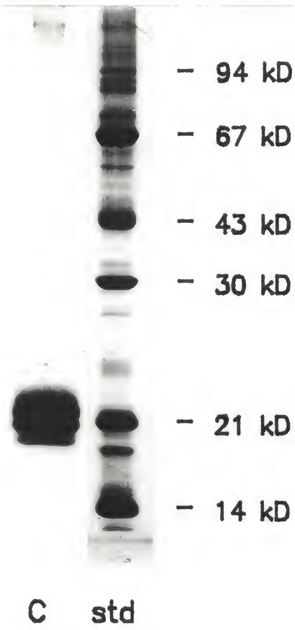


Figure 26. SDS-PAGE patterns of the eluted rock shrimp protein "C" and MW standard proteins.

Table 6. Screening of hybridoma master cultures by ELISA for reactivity with protein "C" and various shrimp extracts.

	Optical density reading at 405 nm[a]			
		Crude shrimp extracts[b]		
Master culture ID	Protein "C"	Pink	White	Rock
4C7	0.475	0.086	0.142	0.400
2C11	1.573	0.099	0.136	0.161
4H1	1.988	0.065	0.059	0.101
2H3	0.670	0.103	0.144	0.207
1D3	0.723	0.095	0.126	0.150
2E8	1.374	0.120	0.093	0.306
1E1	0.427	0.065	0.059	0.101
4H2	1.001	0.176	0.158	1.456

[a] Mouse IgG at 5 µg/mL and 1% BSA were used respectively as positive and negative controls whose respective O.D. readings were >3.0 and 0.061.

[b] Crude shrimp extracts were prepared according to the method described by An et al. (1988) and tested at 5 µg/mL by ELISA. Pink and white shrimp extracts were served as negative controls.

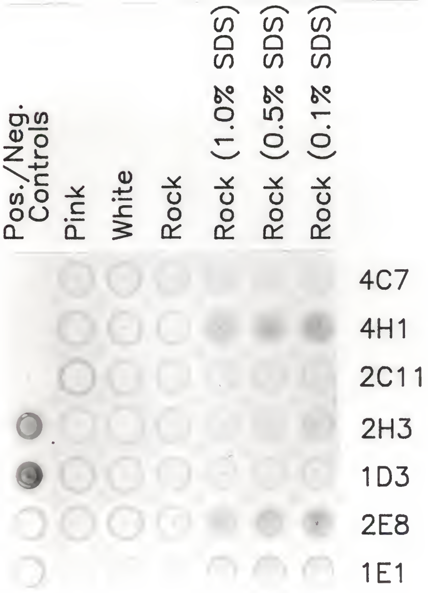


Figure 27. Screening of the uncloned hybridoma cultures by immunodot blotting for reactivity with rock shrimp extracts added with SDS. Pink and white shrimp were used as negative controls, while mouse IgG was used as positive control. Culture 4H2 which showed similar results as 4H1 and 2E8 was not included in this picture.

shrimp extracts containing 0.1, 0.5 or 1.0% SDS (w/v, final concentration). The reactivity was the highest with the rock shrimp extract containing 0.1% SDS as determined by color development, and followed by those with 0.5 and 1.0% SDS (Fig. 27). Dimitriadis (1979) has previously demonstrated that SDS, at concentrations greater than 0.2%, inhibits antigen-antibody reactions to a significant degree in immunodiffusion and immunoprecipitation tests.

The binding of proteins to nitrocellulose membrane is affected by several factors, such as pH, molecular charge, and the presence of competing substances for binding (Wallis et al., 1979). A high concentration of SDS is believed to reduce the ionic interaction of proteins to anionic nitrocellulose membrane by contributing negative charges to the protein molecules. Thus, fewer protein molecules are bound to the nitrocellulose membrane for reaction with the antibody. SDS at a higher concentration also inhibits antigen-antibody reactions by denaturing the protein molecules (Dimitriadis, 1979). The presence of only 0.1% SDS in rock shrimp extract, however, may cause slight denaturing changes in protein "C" configuration for enhanced reactivity with the antibodies produced by these three cultures. Based on the results of the ELISA and immunodot blot tests, the three cultures, 2E8, 4H1, and 4H2, were chosen for cloning.

Only the clones containing a single colony, as observed under an inverted microscope, were screened for reactivity with rock shrimp extract and protein "C" using ELISA and immunodot blot tests (Fig. 28 and Table 7). Only the immunodot blot test results of the clones derived from the 4H2 culture were shown in Fig. 28. The clones only showed high reactivity with rock shrimp extract containing 0.1% SDS. No reactivity occurred with the pink or white shrimp extracts.

The ELISA test results indicated that except for 4H2-11G5, all clones derived from 2E8, 4H1 and 4H2 cultures showed high reactivity with protein "C" (O.D. readings: 0.41-2.0, Table 7). However, the reactivity of the supernatants from these clones with rock shrimp water extracts was inconsistent. For examples, the 4H2-10D3 clone had an O.D. reading of 0.907; whereas, the 2E8-1F5 only had a reading of 0.166 (Table 7). Some clones, such as 4H1-9G6 and 4H2-11G5, were found to have a higher O.D. reading for pink and white shrimp extract, respectively, than for rock shrimp. These two clones may still contain a mixture of hyridoma cells capable of secreting immunoglobulin with different specificities as indicated by Goding (1983).

The results also showed that most clones, especially those derived from the 4H1 and 2E8 cultures, had much higher O.D. readings for protein "C" than for rock shrimp extract. For example, the 4H1-8D9 clone had a reading of 2.040 for protein "C" but only 0.385 for rock shrimp extract (Table 7). Since protein "C" was used to immunize the mice, the McAbs produced this way would be expected to have a higher reactivity for protein "C" than for rock shrimp extract. Heat treatment can cause protein denaturation and exposure of some

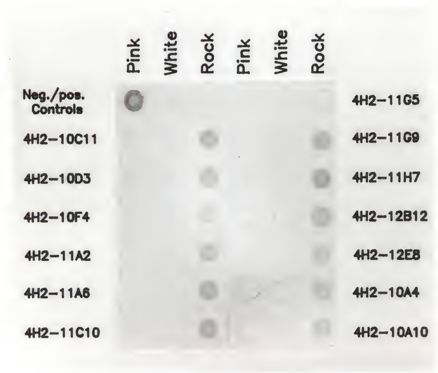


Figure 28. Screening of the monoclones derived from the 4H2 master culture by immunodot blotting for reactivity with shrimp extracts containing 0.1% SDS. Mouse IgG and BSA were used as positive and negative controls, respectively.

Table 7. Screening of cloned hybridoma cultures by ELISA for reactivity with protein "C" and various shrimp extracts.

	Optical density reading by ELISA[a]			
		Crude shrimp extracts[b]		
Master culture ID	Protein "C"	Pink	White	Rock
2E8-1E6	0.461	0.125	0.152	0.229
2E8-1F5	0.552	0.140	0.140	0.166
2E8-1F11	0.535	0.136	0.152	0.234
2E8-1G4	0.501	0.131	0.149	0.217
2E8-2B8	0.518	0.186	0.188	0.333
2E8-2B10	0.579	0.134	0.147	0.438
2E8-2C7	0.528	0.133	0.145	0.224
2E8-2C8	0.447	0.170	0.190	0.311
2E8-2G12	0.405	0.186	0.199	0.300
2E8-2H6	0.549	0.132	0.145	0.228
2E8-2H11	0.522	0.126	0.142	0.234
2E8-3B8	0.452	0.118	0.125	0.197
2E8-3C11	0.509	0.138	0.140	0.225
4H1-7B5	1.813	0.174	0.279	0.404
4H1-7D6	1.819	0.119	0.155	0.351
4H1-8A5	1.868	0.110	0.125	0.463
4H1-8C10	1.917	0.152	0.209	0.364
4H1-8D3	2.026	0.144	0.211	0.377
4H1-8D9	2.040	0.169	0.247	0.385
4H1-8E10	1.744	0.114	0.140	0.308
4H1-8F11	1.816	0.128	0.156	0.365
4H1-9A1	1.722	0.106	0.122	0.251
4H1-9D1	1.929	0.116	0.119	0.248
4H1-9D1	1.839	0.118	0.148	0.379
4H1-9E12	2.057	0.085	0.097	0.210
4H1-9F1	2.003	0.092	0.094	0.216
4H1-9G6	1.844	0.410	0.106	0.235
4H1-9G7	1.790	0.084	0.106	0.246
4H2-10A4	0.527	0.085	0.095	0.371
4H2-10A10	0.845	0.089	0.105	0.832
4H2-10C11	0.680	0.096	0.100	0.812
4H2-10D3	0.706	0.095	0.110	0.907
4H2-10F4	0.899	0.090	0.100	0.249
4H2-11A2	0.845	0.087	0.256	0.405
4H2-11A6	0.993	0.085	0.091	0.575
4H2-11C10	0.744	0.084	0.433	0.786

Table 7. (Continued)

	Optical density reading by ELISA[a]			
		Crude shrimp extracts[b]		
Master culture ID	Protein "C"	Pink	White	Rock
4H2-11G5	0.068	0.078	0.336	0.191
4H2-11G9	0.573	0.083	0.094	0.500
4H2-11H7	0.627	0.079	0.105	0.595
4H2-12B12	0.527	0.099	0.106	0.965
4H2-12E8	0.571	0.099	0.100	0.356

[a] Mouse IgG at 5 μg/mL and 1% BSA were used respectively as positive and negative controls whose respective O.D. readings were >3.0 and 0.061,

[b] The shrimp extracts were prepared according to the method described by An et al. (1988) and tested at 5 μg/mL by ELISA.

antigenic sites that may not be evident when the protein is in its native form (Foegeding, 1988). Thus, McAb specific for heat-induced structure may not react as well with the protein molecule in its native state (Benjamin et al., 1984). The presence of more antigenic determinants on protein "C" than in rock shrimp extracts, as well as the interference by the other antigenically related proteins in the water extract may also account for the higher reactivity of the McAb with protein "C" than with crude rock shrimp extract.

Although several positive clones were selected from each of the three cultures, only one clone from each culture showing the highest reactivity for rock shrimp extract (2E8-2B10, 4H1-8F11 and 4H2-10D3) was chosen and propagated intraperitoneally in mice to produce McAb-containing ascites. The McAbs produced by these three clones all belonged to the $IgG_1(k)$ subclass as determined by the isotyping kit (data not shown). After purification by affinity chromatography, the McAbs were assessed again for reactivity with rock, pink and white shrimp extracts by immunodot blot; the 4H2-10D3 McAb showed the highest specific reactivity for rock shrimp extract (data not shown). This McAb was therefore used to develop immunochemical assays to detect rock shrimp.

This study also illustrates the importance of using both protein "C" and rock shrimp extract during the screening process to obtain a desirable hybridoma clone with a high specificity. This is especially important for the development of a McAb for food component identification. The 4H1-9E12 clone which showed the highest reactivity among all the clones for protein "C" (O.D. reading: 2.057) only showed a weak reactivity for rock shrimp extract at 0.210 (Table 7). On the other hand, the 4H2-10D3 clone which had a reading of only 0.706 for protein "C" had an increased reading of 0.907 for rock shrimp protein extract. Clone 4H1-9E12 would have been selected if only protein "C" was used during the screening process. However, the 4H2-10D3 clone became the top choice when the reactivity of this McAb with both protein "C" and rock shrimp extract were considered.

The use of different screening methods could affect the reactivity of McAbs with a specific antigen. The 4H2-12B12 McAb showed the highest reactivity for rock shrimp extract by ELISA (O.D.: 0.965, Table 7). However, the reactivity of this McAb with the same antigen on immunodot blot was not as high as the 4H2-10D3 McAb as judged by the intensity of the developed color (Fig. 28). The different protein binding mechanism to immunosorbent materials, such as microtitration plates and nitrocellulose membranes could contribute to this variation. Protein binding to nitrocellulose membranes can induce partial denaturation of the protein due to ionic interaction with the surface negative charge (Towbin and Gordon, 1984; Van Oss et al., 1987). Thus the McAb specific for denatured proteins will be more reactive with the partially denatured proteins present on nitrocellulose membranes than with the same proteins in native state bound on ELISA plates.

Specificity Determination of 4H2-10D3 McAb by Western blotting and Immunodot Blotting

The specificity of the 4H2-10D3 McAb for protein "C" and rock shrimp extract was further confirmed by Western blotting (Fig. 29). The specific reaction between shrimp protein components and antibodies could be more clearly assessed using this method (Burnette, 1981; Gershoni and Palade, 1983). Two identical electroblots were produced from an SDS-PAGE gel. One blot was stained for total proteins by Coomassie blue and the other immunostained using the 4H2-10D3 McAb. The immunostained blot showed only one positive area of reaction corresponding to the protein "C" position on the electroblot stained with Coomassie blue. This result suggested that the 4H2-10D3 McAb reacted only with the protein "C" component of rock shrimp and not with the proteins from pink or white shrimp, or with the MW protein standards.

Cross-reaction of McAbs may occur with the unrelated antigens which share a structural similarity with the immunizing antigen (Goding, 1983). To determine if any cross-reactivity occurred, the 4H2-10D3 McAb was tested by immunodot blot assay against 23 commonly consumed unrelated species of seafood and meat (clam, oyster, sea scallops, chicken, pork, beef, fishes, lobsters, 10 different shrimp from various regions) as well as the three rock shrimp from the East and West coasts of Florida (Port Canaveral and Cedar Key) at two different seasons, February and December (Fig. 30a and b). The McAb showed similar reactivity with rock shrimp

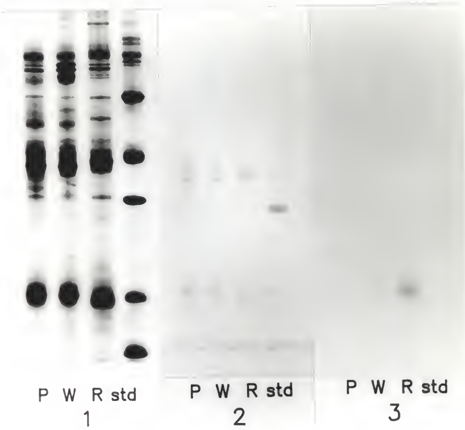
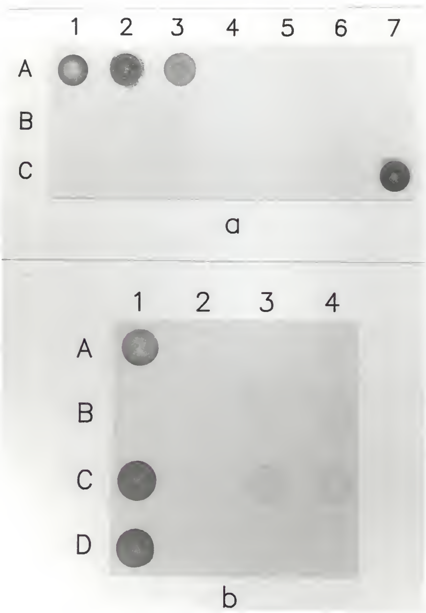


Figure 29. Determination of the specificity of 4H2-10D3 McAb in reacting with rock, pink and white shrimp as well as MW protein standards as analyzed by the Western blotting technique. (a) The SDS-PAGE pattern of pink (P), white (W) and rock (R) shrimp as well as the MW protein standards (std). (b) The Coomassie brilliant blue-stained electrophoretic blots of pink, white and rock shrimp as well as the MW protein standards. (c) The 4H2-10D3 McAb-immunostained electrohoretic blot of pink, white and rock shrimp as well as the MW protein standards. Only the protein "C" of rock shrimp reacted with 4H2-10D3 McAb and showed blue color on immunostained electrophoretic blot.

Figure 30. Determination of the specific reactivity of 4H2-10D3 McAb with rock shrimp as analyzed on immunodot blot with the water extracts of (a) various shrimp and lobsters as well as (b) various seafood and meat. The samples used in test (a) include A1, mouse IgG; B1, 1% BSA; A2, rock shrimp from Port Canavarel, FL; A3, rock shrimp obtained in February from Cedar Key, FL; A4, white shrimp from Ecuador; A5, Argentine-red shrimp from Argentine; A6, blue shrimp from Ecuador; B2, pink shrimp from Tampa, FL; B3, white shrimp from Peru; B4, brown shrimp from North Carolina; B5, white shrimp from Columbia; B6, white shrimp from Georgia; C2, white shrimp from Honduras; C3, blue shrimp from Honduras; C4, Lobster from Australia; C5, Lobster from Florida; C7, rock shrimp obtained in December from Cedar Key, FL. The samples used in test (b) include A1, mouse IgG as positive control; B1, 1% BSA as negative control; C1, rock shrimp from Port Canaveral, FL; D1, rock shrimp from Cedar Key, FL; A2, clam; B2, oyster; C2, sea scallop; D2, trout; A3, salmon; B3, flounder; C3, tuna; D3, grouper; A4, beef; B4, pork; C4, chicken.


1
2
3
4
5
6
7
A
B
C
a
1
2
3
4
A
B
C
D
b

samples from different locations (dots A2 and A3 in Fig. 30a; and C1 and D1 in Fig. 30b) and during different seasons (dots A3 and C7 in Fig. 30a), indicating that this McAb was highly specific for rock shrimp regardless of the harvest location and season. No cross-reactivity was observed with the other shrimp species or lobsters. The results also indicated that the rock shrimp-specific McAbs produced by the hybridoma technique is very effective to differentiate the slight difference among the protein "C"-like proteins present in all other shrimp species (Fig. 23). A very slight background color developed for the distant species samples including salmon, beef, pork and chicken. This may indicate the presence of protein "C"-like molecules in these distant species. However, the intense blue color developed for rock shrimp was easy to differentiate from this slight background. The 4H2-10D3 McAb is thus highly specific for for rock shrimp.

Optimization of ELISA and Immunodot Blot Tests

Pretreatment of antigens. Studies with immunodot blot and ELISA indicated that the 4H2-10D3 McAb could react with rock shrimp extract in the native state. Heat treatment of rock shrimp extracts in boiling water for 5 min greatly enhanced the reactivity with this McAb as shown by the immunodot blot (Fig. 31) and the ELISA (Fig. 32) tests. Since protein "C" used to immunize mice had been heat-denatured before the SDS-PAGE run, it is not surprising that the 4H2-10D3 McAb would be more reactive to heat-treated rock shrimp extract than to a non-treated extract. Heat treatment causes

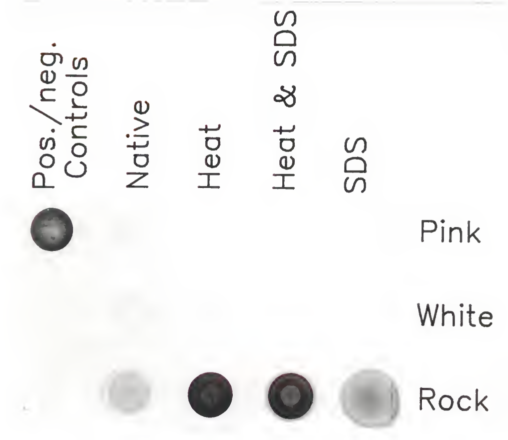


Figure 31. Effect of the heat treatment, addition of SDS or the combination of both on the reactivity of rock shrimp extract with 4H2-10D3 McAb as analyzed by immunodot blot.

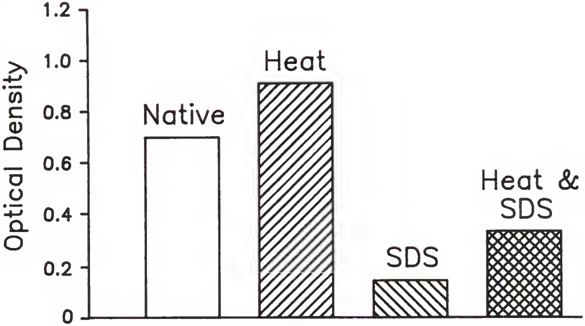


Figure 32. Effect of heat treatment, the addition of SDS or the combination of both on the increment of reactivity of rock shrimp proteins with 4H2-10D3 McAb on ELISA.

protein conformational changes and exposes antigenic determinants with which this McAb reacts. The high specificity of this McAb for heat-denatured proteins enables its use to detect the presence of rock shrimp in processed seafood products.

The addition of SDS to heat-treated or non-treated rock shrimp extract also increased antigen reactivity with the 4H2-10D3 McAb on the immunodot blot, but to a lesser extent than heat treatment of the antigen alone (Fig. 31). The increase in reactivity in the presence of low concentration of SDS was believed to be related to protein conformational changes caused by SDS as previously discussed. SDS might also reduce the amount of heat-denatured proteins available for reaction with McAb by affecting the binding of the former to the nitrocellulose membrane. It has been reported that the presence of low concentrations of SDS did not affect antibody production or their specificity, but decreased the nonspecific binding of antibody to unrelated antigen (Dimitriadis, 1979; Stumph et al., 1974; Tjian et al., 1974).

The reactivity of the 4H2-10D3 McAb with the native or heat-treated antigen determined by ELISA was reduced after SDS was added to the antigen (Fig. 32). Protein binding to plastic microtitration plates is achieved by adsorption through hydrophobic interactions between nonpolar protein substructures and the nonpolar plastic matrix (Clark and Engvall, 1980). Because the presence of interfering chemicals, such as SDS, could affect this weak binding

(Engvall, 1980), the adsorption of shrimp antigen onto ELISA plates was reduced, thus reducing the reactivity of the rock shrimp with the McAb.

Optimization of antibody and antigen concentrations for the ELISA test. The reactivity of the 4H2-10D3 McAb with rock shrimp extract at 5 μg/mL increased as the amount of McAb used was increased; the reactivity plateaued when the antibody concentration reached 20 μg/mL (Fig. 33). The reactivity of this McAb with pink or white shrimp extract was negligible (O.D. readings less than 0.11) even with the increased amount of McAb. To ensure high reactivity, McAb at 50 and 100 μg/mL was used to determine the optimized antigen concentration for reaction.

Rock shrimp extract at 10 μg/mL showed the highest reactivity with the 4H2-10D3 McAb at 50 or 100 μg/mL (Fig. 34). This "prozone" effect could be due to the limitation of the availability of the binding sites for the antigen on the plastic surface. The surface approaches saturation when a high concentration of antigen was added (Douillard and Hoffman, 1983; Pesce et al., 1981); the optimal range of antigen coupled to microtitration plates was estimated to be within 1-50 μg/mL (Douillard and Hoffman, 1983). In addition, proteins at high concentration may aggregate and reduce the number of antigenic determinants available for antibody binding. For a better ELISA result, rock shrimp extract as well as the unknown samples at 10 μg/mL was used to react with the McAb.

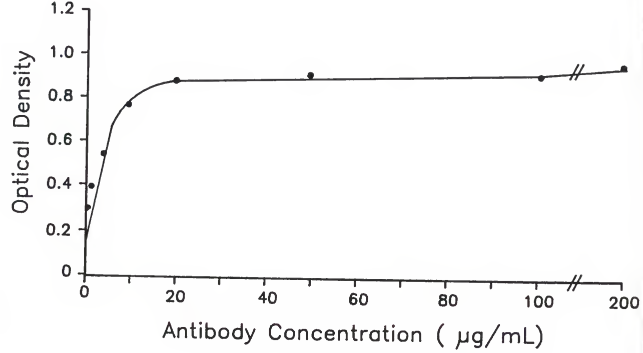


Figure 33. Dose-related changes of the reactivity of 4H2-10D3 McAb with rock shrimp extract at 5 μg/mL by ELISA.

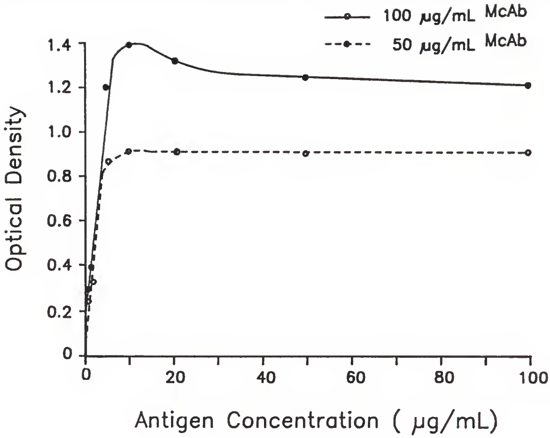


Figure 34. Dose-related changes of the reactivity of rock shrimp extract with the 4H2-10D3 McAb at 50 and 100 µg/mL by ELISA.

It was also noted that the use of the antibody at 50 μg/mL generally produced a lower absorbance reading than at 100 μg/mL. This effect was more significant at an antigen level of less than 10 μg/mL. Antibody binding to antigen-coated immunosorbent matrix has been reported to be proportional to antibody concentration (Pesce et al., 1977); thus the higher the McAb concentration is used, the better the McAb binding to the antigen coated on the microtitration plate. Therefore McAb at 100 μg/mL was used to ensure the highest sensitivity of the ELISA test for the detection of or quantitation of rock shrimp.

Determination of the Accuracy of the McAb in Identifying Rock Shrimp in a Blind Study Using the ELISA Test

Rock shrimp samples obtained from the East and West coasts of Florida (samples X8 and X7) and at different months (February and December; samples X7 and X22) were correctly identified in a blind study using 24 seafood and meat samples (Table 8). Rock shrimp samples obtained from different locations and at different seasons were equally reactive with the McAb. The reactivity for rock shrimp samples was 9 times higher than that of the other seafood and meat samples, or the other unrelated shrimp samples. Since the yellow color developed by ELISA for rock shrimp was so intense, the reactivity could be detected by direct inspection of the plates without the use of an ELISA reader. This will prove advantageous when using these antibodies for field testing.

Table 8. The reactivity of 4H2-10D3 McAb[a] with the water extracts[b] of the various seafood and meat samples in the blind study using the ELISA test.

Sample No.	Unknown sample	Optical density[c]
X1	Trout	0.111 ± 0.012
X2	Sea scallops	0.095 ± 0.009
X3	Argentine-red shrimp, Ecuador	0.095 ± 0.011
X4	Beef	0.097 ± 0.004
X5	Salmon	0.126 ± 0.002
X6	Flounder	0.102 ± 0.008
X7	Rock shrimp, Cedar Key, FL (February)	1.508 ± 0.068
X8	Rock shrimp, Port Canaveral, FL	1.125 ± 0.071
X9	White shrimp, Columbia	0.112 ± 0.016
X10	White shrimp, Honduras	0.109 ± 0.006
X11	Oyster	0.098 ± 0.012
X12	Tuna	0.117 ± 0.002
X13	Blue shrimp, Honduras	0.093 ± 0.010
X14	Lobster, Australia	0.094 ± 0.011
X15	White shrimp, Peru	0.099 ± 0.015
X16	Clam	0.096 ± 0.004
X17	Grouper	0.102 ± 0.008
X18	Pork	0.098 ± 0.007
X19	Blue shrimp, Ecuador	0.097 ± 0.011
X20	Lobster, FL	0.094 ± 0.011
X21	Brown shrimp, NC	0.097 ± 0.010
X22	Rock shrimp, Cedar Key, FL (December)	1.303 ± 0.059
X23	Pink shrimp, FL	0.096 ± 0.012
X24	Chicken	0.104 ± 0.019
X25	White shrimp, Ecuador	0.112 ± 0.013
X26	White shrimp, GA	0.106 ± 0.014

[a] The McAb used was 10 μg protein/well.
[b] The protein extracts used was 1 μg/well.
[c] Data are means ± standard deviations.

Use of the McAb to Quantitate Rock Shrimp in Mixtures in a Blind Study Using the ELISA test

When used in the ELISA, the 4H2-10D3 McAb could accurately identify those unknown samples which contained various amounts of rock shrimp. Among the 24 unknown samples prepared by randomly mixing the various seafood, fish or meat samples in various ratios with rock shrimp (Table 4), all 13 samples which contained rock shrimp were correctly identified by the ELISA. The O.D. readings of the positive samples were all greater than 1.0 (data not shown).

The ability of the McAb to detect the lowest level of rock shrimp present in mixture samples deterined with the fivefold serial dilutions of the samples. The detectability was interpreted as the minimal amount of rock shrimp protein present in the most diluted samples that had an ELISA O.D. reading greater than 0.2, a value at least two times greater than the O.D. readings of the background and negative controls. The amount of rock shrimp protein present in diluted samples was calculated by multiplying the initial total protein content of the unknown samples times the dilution factor and the percent content of rock shrimp in the original sample mixture. The detectability was found to range from 1.9 to 136 ng with an average of 43 ng (Table 9). The high variability of the values was partially attributed to the variable protein levels that the minimal O.D. readings were chosen from. Rock shrimp proteins in highly diluted samples bound better to the microtitration plates, perhaps due

Table 9. Sensitivity limit of the ELISA test in determining rock shrimp content in mixture samples.

Positive samples[a]	Absorbance	Dilution range	Protein content (μg)	Rock shrimp percentage	Rock shrimp content[b] (μg)
Z4	0.229	5^{-7}	250	100	0.0032
Z5	0.219	5^{-5}	250	100	0.08
Z13	0.325	5^{-5}	250	100	0.08
Z15	0.342	5^{-7}	500	33	0.021
Z16	0.295	5^{-5}	500	33	0.053
Z17	0.278	5^{-5}	500	33	0.053
Z18	0.281	5^{-5}	500	33	0.053
Z19	0.203	5^{-6}	500	33	0.011
Z20	0.263	5^{-6}	417	60	0.016
Z21	0.250	5^{-6}	333	33	0.007
Z22	0.393	5^{-4}	500	17	0.136
Z23	0.263	5^{-7}	500	30	0.0019
Z24	0.303	5^{-5}	333	40	0.042

[a] Positive mixture samples containing rock shrimp as determined in the blind study by ELISA.

[b] Rock shrimp content was calculated by multiplying protein content with the dilution range and rock shrimp percentage in the mixture.

to reduced competition by other proteins for the same binding sites. The composition of the sample mixtures containing seafood and/or meat proteins also could affect the reactivity of rock shrimp extract with the McAb.

Since protein "C" accounted for 19.7% of total rock shrimp protein, the sensitivity of the 4H2-10D3 in detecting protein "C" using ELISA was 8.4 ng (43 ng X 19.7%). This was very close to the result of the other study in which 2.5 ng protein "C" was found to be the minimal amount that showed an ELISA O.D. reading greater than 0.2 when reacted with the 4H2-10D3 McAb (data not shown). Therefore rock shrimp proteins within a nanogram range could be detected using this ELISA assay to reveal the presence of rock shrimp in mixtures containing unrelated species of seafood or meat.

Conclusion

McAbs have been produced using hybridoma technology which are specific for unique rock-shrimp proteins of MW 17.7 and 18.5 kD (protein "C"). These antibodies could be used in immunoassays, such as ELISA and immunodot blots, to detect and/or quantitate the presence of rock shrimp in samples containing mixtures of diverse seafoods or other non-seafood meats. The immunoassays are fast, sensitive and reproducible. They can be used for fast screening of species used in food products. This approach should prove useful for detection of specific components in food products.

CHAPTER VIII
SUMMARY AND RECOMMENDATIONS

Water and 1% SDS were shown to be the more effective protein extractants for raw and cooked shrimp, respectively. The use of the proper extraction system not only increased protein extractability but also improved the accuracy of shrimp species identification by SDS-PAGE or IEF. This is particularly important for identification of the species origin of cooked seafood because heating causes protein denaturation and aggregation. The use of protein solubilizer, such as SDS, improves protein extractability.

The use of urea at 9.2 M in the isoelectric focusing gel was found to greatly increase protein resolution possibly by reducing hydrophobic interactions. Many more protein bands were resolved on this gel than on the native IEF gels containing no urea. Protein separation can further be enhanced by using an ampholyte mixture containing 4 parts of pH 4.5-6 and one part of pH 3-10 ampholytes. The use of the narrow pH range ampholytes expands the distance between protein bands, and thus makes the identification of closely related shrimp species easier. Using these optimized conditions, correct species identification of raw or cooked shrimp both native or in a mixture was achieved.

Electrophoretic identification method can be replaced by fast and accurate immunochemical assays using monoclonal antibodies (McAbs) specific for seafood species. McABs specific for rock shrimp were developed from a rock shrimp-specific component, protein "C", and used to detect this seafood species by immunoassays. One McAb, 4H2-10D3, was shown to be highly specific for rock shrimp with negligible cross-reactivity with other unrelated seafood or meat samples. This McAb could be used for both ELISA and immunodot blot to identify rock shrimp or to detect the presence of rock shrimp in various seafood and meat mixtures. This McAb was demonstrated by ELISA to be highly sensitive in detecting rock shrimp protein at a level of as low as 43 ng.

Protein "C" is unique in that it is relatively specific for rock shrimp and is not present in the other species of seafood and meat. Protein "C" is heat resistant; heat treatment in boiling water for 5 min did not affect its mobility on the SDS-PAGE gels or antigenic reactivity with the 4H2-10D3 McAb. The unique nature of this protein makes it an effective marker for identification of rock shrimp in heat-processed food products. Antibodies developed to this protein recognized both native and heat-denatured forms of protein "C". Therefore, the use of one type of antibody developed this way can possibly identify the species of raw seafood and heat processed products.

It is interesting to study the nature of protein "C" and its relationship to the other proteins found in different shrimp species which have molecular weight similar to that of protein "C". Protein "C" can be separated from the other rock shrimp proteins using an affinity column conjugated with protein "C"-specific McAbs. From the determination of the extractability of protein "C", an accurate estimation procedure to quantitate rock shrimp in mixtures can be established. The protein content in cells as well as the level of enzymes used for the synthesis of proteins, chitin, lipids, and nucleic acids needed for molting are known to change with the molting stage. Since muscle protein levels are affected by the sex, age and molting stage of the crustacea, it will be interesting to investigate the quantitative and qualitative relationship between the aforementioned factors on the protein "C" of rock shrimp and the similar proteins present in other shrimp species.

It would be possible to develop an easy-to-use species detection kit applying immunodot blotting after the specific McAbs for some popular or prevalent seafood species are produced. The species-specific McAb can be fixed in the immunodot blot in a container and used to react with crude protein extracts of various seafood products. From the color development following the addition of enzyme-labeled antibodies and substrates, the antigen-antibody reaction can be demonstrated. This test procedure has the advantage over the ELISA in field testing by reducing the equipment requirement. This assay method can be used not only for detection but also for quantitation of a particular species by including various amounts of reference species fixed on the blot.

APPENDIX
ELECTROPHORETIC IDENTIFICATION OF FISH SPECIES USED IN SURIMI PRODUCTS

Introduction

Surimi is a mechanically deboned, minced fish meat (washed to remove blood, fat, soluble pigments and other odoriferous substances) which is mixed with cryoprotectants, namely, sugar and/or sorbitol (Lee, 1984, 1986a). The washing process greatly improves the color and the odor of the minced fish and stabilizes the functional properties of surimi during frozen storage (Rasekh et al., 1980). Due to its light color, bland odor and unique gelling properties, surimi is used as a functional protein ingredient in the manufacture of a variety of fabricated seafood products such as crab legs, scallops, lobster and shrimp analogs (Lanier, 1986; Regenstein and Lanier, 1986). Concurrent with the development of surimi processing technology, surimi-based products have recently demonstrated tremendous growth and acceptance in the US marketplace.

To formulate the fabricated seafood products, surimi is combined with natural shellfish meat, shellfish flavoring agents, salt, water and starch, and/or egg white to modify the functionality and to enhance the flavor and texture. The finished products must be labeled properly to meet the Food and Drug Administration (FDA) guidelines which reflect the nature of the products. Martin (1986) has also stressed the need to establish proper nomenclature and labeling for newly developed seafood analogs. These must meet FDA requirements which have an impact on the two major ingredients in the fabricated seafood products: the fish species as the main ingredient and the other species, such as snow crab meat for crab meat analog, as the additional ingredient. One area of concern is a blended surimi in which the surimi make-up is more than one species. The use of such a blended surimi is likely to increase as industry is trying to make the production more economical. Problems also exist regarding the labeling of the content of the specific seafood components. Products with claims of 35% crab meat are widely sold when the use of over 10% crab meat is known to show detrimental effect to the product (Regenstein and Lanier, 1986). Thus the establishment of useful methods to identify species origin, and if possible, the content of surimi product is of great need for regulatory purposes and for protection of consumer's values. Compared to raw fish, the identification of the fish origin in finished surimi-based products is complicated by the fact that the fish proteins are heat-denatured during steaming, and that most of the sarcoplasmic proteins are washed away during the rinsing process (Lee, 1984, 1986a). Using 1% sodium dodecyl sulfate (SDS) or 8 M urea to extract proteins, An et al. (1988, 1989a) have successfully identified the species and genus of cooked shrimp on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and urea gel isoelectric focusing (IEF). However, no attempts have been made on the species identification in surimi samples. This study was therefore carried out to determine if the above mentioned techniques could effectively differentiate the protein patterns of Alaska pollock and red hake and to identify these two species used in surimi.

Materials and Methods

Alaska pollock (Theragra chalcogramma) and red hake (Urophycis chuss), the two most commonly utilized species in surimi manufacturing (Babbitt, 1986), were used. Red hake fillet and surimi were prepared at the surimi pilot plant of the Department of Food Science and Nutrition at the University of Rhode Island and were shipped frozen to the Food Science and Human Nutrition Department at the University of Florida. Red hake surimi was prepared following a procedure described by Lee (1986b). Frozen Alaska pollock fillet was provided by Arctic Alaska Seafoods, Inc. (Seattle, WA), while surimi was manufactured by the Alaska Pacific Seafoods (Kodiak, AK) and obtained from the Alaska Fisheries Development Foundation (Anchorage, AK). The samples were cut into small pieces of about 5 g, put in a Whirl-Pak bag (Fisher Scientific), and stored at -33°C until needed. Heat treatment of the samples was done by cooking in boiling water for 5 min. All experiments were repeated at least twice.

Protein Extraction and Sample Preparation for Gel Electrophoresis

Raw fish fillet and surimi samples were extracted with water only, while cooked samples were extracted with water, 1% SDS (95% purity) or 8 urea (electrophoresis purity reagent). All solvents contained 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM EDTA and 0.01% (w/v) sodium azide to inhibit proteases and microbial growth. The chopped samples of the fish fillet or surimi were combined in a beaker with each solvent at a ratio of 1:3 (w/v), and homogenized at room temperature for 1 min using a Polytron (setting 5.5). The homogenates were centrifuged at 48,000 x g for 20 min at 20°C, the supernatants were collected, and the protein contents determined (Lowry et al., 1951). The protein content in the supernatant was then adjusted with water to 5 μg/μL. Sucrose (3-4 granules) was then added to each 100 μL extract, and 100 μg protein was applied on a IEF gel. For SDS-PAGE, 35 μg protein was loaded on the gel after the protein content of the supernatant was adjusted to 1.75 μg/μL with Tris-HCl buffer (pH 6.8) and SDS solution, heat-denatured, then finally sucrose and bromophenol blue were added (O'Farrell, 1975).

The fish and surimi extracts were mixed in a 1:1 protein ratio (w/w, mixed sample). The mixture was processed as above and subjected to SDS-PAGE or IEF analysis.

Isoelectric Focusing

A gel mixture containing 4% (w/v) acrylamide, 2% (w/v) Triton X-100 and 9.2 M urea was heated to 37°C with shaking for 5 min to dissolve urea. Ampholyte was added at a final concentration of 6.2% and the gel mixture was degassed for 5 min. The mixture was then combined with fresh persulfate solution and N,N,N',N'-tetramethylethylenediamine (TEMED) (final concentration: 0.02% (v/v) and 0.14% (v/v), respectively), and poured into 16 x 20 cm slab gel plates (0.75 mm thick) assembled with a comb. The gel was allowed to polymerize for 1 hr. Lysis buffer containing 9.5 M urea, 2% Triton X-100 and 2% (v/v) ampholyte was overlayed on the gel following the removal of the comb. The gel was allowed to sit for another hour. Prefocusing of the gel was done, after the lysis buffer was replaced with a fresh buffer, at 200 V for 15 min, 300 V for 30 min, and then 400 V for 30 min in a Protean II electrophoresis slab gel unit using 0.01 M phosphoric acid as the anode solution and 0.02 M sodium hydroxide as the cathode solution.

Both the lysis buffer and cathode solution were removed after prefocusing. Following the application of protein samples to the gel, an aqueous solution containing 2% (w/v) Triton X-100 and 2% ampholyte was overlayed on the top of the protein samples. After the gel plate was reassembled in the electrophoresis unit, fresh cathode solution was added to the chamber. Proteins were focused at room temperature for 17 hrs at 400 V with circulating water. After the IEF run, the gel was stained with Coomassie blue R-250 and destained.

The apparent pI values of the fish and surimi proteins were determined indirectly by comparing their R_f values on the gel with those of the protein standards following the previously described procedures (An et al., 1989a). The pH profile of the gel was determined by scanning the pH throughout the gel using a micro-surface pH electrode. A linear relationship of gel pH and the R_f values of protein standards enabled the apparent pI values for the sample proteins to be determined. The protein standards (Broad pI kit, pH 3-10, Pharmacia) contained: trypsinogen, pI 9.30; lentil lectin-basic band, pI 8.65, -middle band, pI 8.45, -acidic band, pI 8.15; horse myoglobin-basic band, pI 7.35, -acidic band, pI 6.85; human carbonic anhydrase B, pI 6.55; bovine carbonic anhydrase, pI 5.85; ß-lactoglobulin A, pI 5.20; soybean trypsin inhibitor, pI 4.55; and amyloglucosidase, pI 3.50.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the modified procedure of Laemmli (1970) and O'Farrell (1975) using a Protean II (vertical slab) unit. Slab gels consisted of a running gel (10.4%) which was polymerized overnight, and a stacking gel (3.1%) which was poured 2 hr before sample application. The protein samples were run initially at a constant current of 15 mA/slab and then increased to

30 mA/slab when the marker front reached the running gel. Following electrophoresis, the proteins were stained with 0.125% (w/v) Coomassie Brilliant Blue R-250 in 40% ethanol and 7% acetic acid, and then destained in 7% acetic acid. Electrophoretic patterns were recorded by developing the positive image using a Kodak Electrophoresis Duplicating Paper (EDP paper). Molecular weights of the protein bands were determined according to the method of Weber and Osborn (1969) and Weber et al. (1972) using a low molecular weight protein kit (Pharmacia) containing phosphorylase b (MW 94 kD), bovine serum albumin (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), soybean trypsin inhibitor (21.1 kD) and α-lactalbumin (14.4 kD). α-Lactalbumin was not included for MW determination due to its high R_f value (>0.90) (Dunker and Rueckert, 1969; Weber and Osborn, 1975).

Results and Discussion

Water Extracts of Intact Fish and Surimi

SDS-PAGE and IEF were shown to be effective in demonstrating species differences in protein patterns of the water extracts of raw fish and surimi samples (Figs. 35 and 36). Using SDS-PAGE, Alaska pollock showed characteristic bands with MW's of 94.9, 89.5, 88.3 and 79.2 kD while the red hake showed specific bands with MW's of 87.0, 68.3, 22.7 and 21.9 kD. All these species specific bands were found in the fish and fish-surimi mixtures (Fig. 35).

A portion of the sarcoplasmic proteins (up to 21% of minced meat was washed away from the fish proteins during

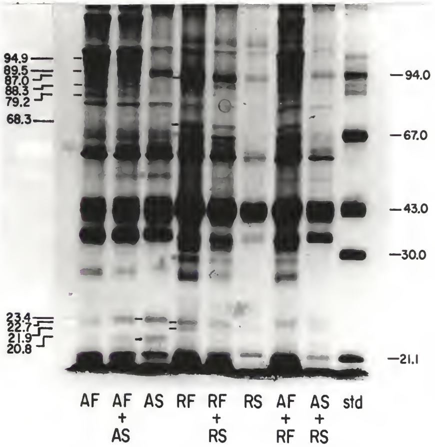


Figure 35. SDS-PAGE patterns of intact fillet and surimi of Alaska pollock and red hake extracted with water. Sample mixtures were prepared as shown by the abbreviations. The numerical designations indicate molecular weights (kD) of the protein bands. AF: Alaska pollock fillet, AS: Alaska pollock surimi, RF: red hake fillet, and RS: red hake surimi.

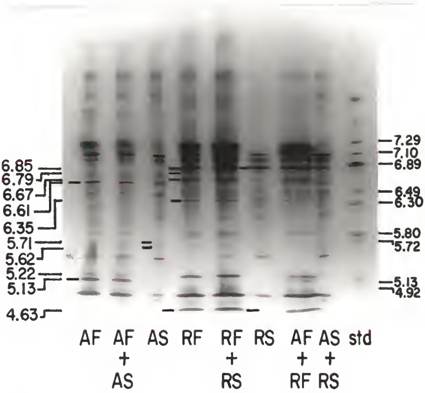


Figure 36. IEF patterns of intact fillet and surimi of Alaska pollock and red hake extracted with water (cathode on top). Sample mixtures were prepared as shown by the abbreviations. The numerical designations indicate apparent pI values of the protein bands. AF: Alaska pollock fillet, AS: Alaska pollock surimi, RF: red hake fillet, and RS: red hake surimi.

surimi manufacturing (Lee, 1986a). This may cause complications in the species identification process of surimi samples, especially when the specific proteins in samples were only present in minor quantities in the fish. Generally, the surimi samples still showed the basic patterns as those of the fish fillet form which they were made. Some of the characteristic protein bands, such as the 94.9, 88.3 and 79.2 kD of Alaska pollock and the 68.3, 22.7 and 21.9 kD of red hake, were missing in the corresponding surimi samples. New bands with MW of 51.3, 23.4 and 20.8 kD were found in Alaska pollock surimi. These bands were also present in the AS+RS mixture and they can be used with reservation to differentiate surimi mixtures. Due to the similarities of the AS and RS banding patterns, the AS+RS mixture patterns can hardly be differentiated as being AS alone or as the combination of AS and RS. However, the use of the IEF patterns, as indicated in the later sections, will help the identification process.

The study with IEF showed that protein bands at the anodic end were more useful for species identification for both the fish and the surimi samples. Alaska pollock had specific protein bands with pI values of 6.61 and 5.13 while the red hake had specific bands of 6.85, 6.79, 6.67, 6.35 and 4.63 (Fig. 36). These specific bands were evident in the fish and fish-surimi mixtures. The 5.22 band of Alaska pollock and red hake, the 5.13 band of Alaska pollock, and the 4.63 band of red hake were missing in the surimi samples. Tow bands with pI values of 5.71 and 5.62 were found in Alaska pollock surimi. The reason for their presence in surimi and not in the corresponding fish is not known. It was speculated that some of the relative percent of the minor proteins had been increased after washing due to the loss of major proteins. The reliability of the surimi supply as well as the presence of residual proteolytic enzyme activity may also contribute to the appearance of the new bands.

The identification of fish species used in surimi was made possible using a urea IEF system. The patterns of AS and RS could be differentiated from each other using the specific bands of 5.71 and 5.62 for AS and 6.85 and 4.63 for RS. These specific bands were also present in the AS+RS mixture; this makes the differentiation of AS, RS, and AS+RS possible.

Water Extraction of Cooked Fish and Surimi

Heat treatment of fish and/or surimi greatly reduced the number of water-extractable protein bands present on the SDS-PAGE and IEF gels (Figs. 37 and 38). Minor bands with MW's of 21.7 and 23.6 kD were specific for the cooked surimi of Alaska pollock and red hake, respectively (Fig. 37). Therefore they could be used for species identification of a surimi mixture (AS+RS) containing Alaska pollock and red hake. The 19.9 kD band was specific for the cooked Alaska pollock fillet and could be used to indicate the presence of Alaska pollock in a fish mixture (AF+RF).

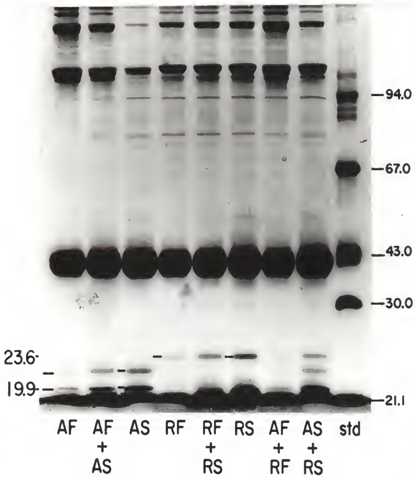


Figure 37. SDS-PAGE patterns of cooked fillet and/or surimi of Alaska pollock and red hake extracted with water. Sample mixtures were prepared as shown by the abbreviations. The numerical designations indicate molecular weights (kD) of the protein bands. AF: Alaska pollock fillet, AS: Alaska pollock surimi, RF: red hake fillet, and RS: red hake surimi.

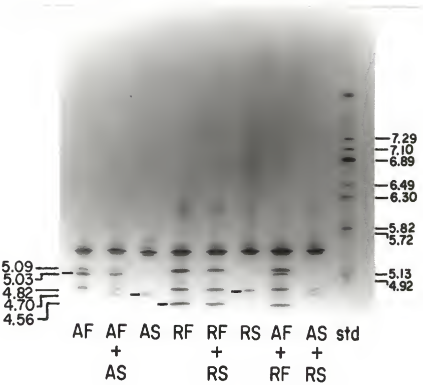


Figure 38. IEF patterns of cooked fillet and/or surimi of Alaska pollock and red hake extracted with water (cathode on top). Sample mixtures were prepared as shown by the abbreviations. The numerical designations indicate apparent pI values of the protein bands. AF: Alaska pollock fillet, AS: Alaska pollock surimi, RF: red hake fillet, and RS: red hake surimi.

The IEF study also indicated that a protein band with a pI of 5.03 was specific for Alaska pollock whereas the one at 4.56 was specific for red hake (Fig. 38). The 5.09 band was detected only in the water extracts of the two cooked fish samples but not in the surimi, whereas the 4.70 band was detected only in cooked Alaska pollock surimi. The 4.82 minor band of the Alaska pollock fillet was not detected in the corresponding surimi sample. Differentiation of AS, RS and AS+RS mixture could be achieved from the presence of the 4.70 band for AS and the 4.82 band for RS. However, washing of various batches of surimi could result in the 4.70 band for AF being present in the surimi. Therefore care must be taken with the water-extracted cooked surimi because of the chance of the doublet for AS+RS resulting from the AF alone.

SDS and Urea Extracts of Cooked Fish and Surimi

As noticed in our previous studies (An et al., 1988, 1989a), the use of SDS and urea greatly enhanced protein extraction from cooked samples than that of water. Fish and surimi also showed an increased number of protein bands on the electrophoretic gels after treating cooked samples with SDS and urea. In addition, the banding patterns of the SDS and urea extracts were very similar on the SDS-PAGE gels. Many of the low MW bands of the SDS-PAGE gels were species-specific and could be used for species identification (Figs. 39 and 40). These SDS-extractable bands were the 47.1, 45.9, 35.5, 23.8 and 21.2 kD bands for Alaska pollock fillet and surimi, and the 25.3 and 20.3 kD bands for red hake fillet

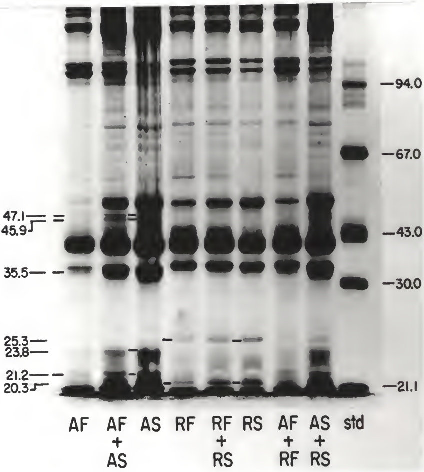


Figure 39. SDS-PAGE patterns of cooked fillet and/or surimi of Alaska pollock and red hake extracted with SDS. Sample mixtures were prepared as shown by the abbreviations. The numerical designations indicate molecular weights (kD) of the protein bands. AF: Alaska pollock fillet, AS: Alaska pollock surimi, RF: red hake fillet, and RS: red hake surimi.

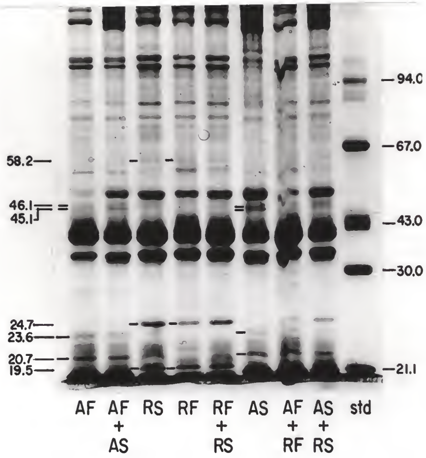


Figure 40. SDS-PAGE patterns of cooked fillet and/or surimi of Alaska pollock and red hake extracted with urea. Sample mixtures were prepared as shown by the abbreviations. The numerical designations indicate molecular weights (kD) of the protein bands. AF: Alaska pollock fillet, AS: Alaska pollock surimi, RF: red hake fillet, and RS: red hake surimi.

and surimi (Fig. 39). For urea extracts, the 46.1, 45.1, 23.6, and 20.7 kD bands, and the 58.2, 24.7 and 19.5 bands were specific for Alaska pollock and red hake, respectively.

From the specific banding patterns and the presence of species-specific protein bands displayed on IEF gels, the species origin of the SDS and urea extracts of fish and/or surimi samples could be achieved (Figs. 41 and 42). The patterns were different between the two fish species and the two surimi samples. More protein bands were extracted by urea from the cooked samples than by SDS or water (Figs. 38, 41 and 42).

The IEF patterns of the SDS extracts revealed that the bands with pI values of 5.72, 5.12 or 4.80 were specific for Alaska pollock surimi, fish fillet, or both, respectively; whereas the bands of 6.85, 6.74, 5.22 and 4.64 were specific for red hake fish sample but not the surimi. The 5.49 band of red hake was specific both for the fish and surimi. Differentiation of AS, RS and AS+RS mixture could be achieved from the presence of the 5.49 band of RS and 4.80 band of AS (Fig. 41). For urea extracts, the protein patterns on the IEF gels were similar between the fish and surimi samples even though some specific bands were missing in surimi samples. The 5.65 band was specific for Alaska pollock, whereas the 5.59 and 5.54 bands were specific for red hake (Fig. 42). The 5.10 and 4.94 bands of the Alaska pollock fish, and the 5.17 and 4.71 bands of the Alaska pollock fish, and the 5.17 and

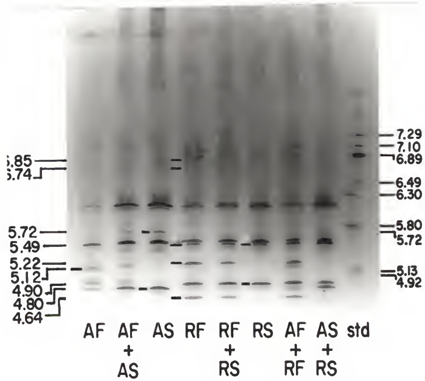


Figure 41. IEF patterns of cooked fillet and/or surimi of Alaska pollock and red hake extracted with SDS (cathode on top). Sample mixtures were prepared as shown by the abbreviations. The numerical designations indicate apparent pI values of the protein bands. AF: Alaska pollock fillet, AS: Alaska pollock surimi, RF: red hake fillet, and RS: red hake surimi.

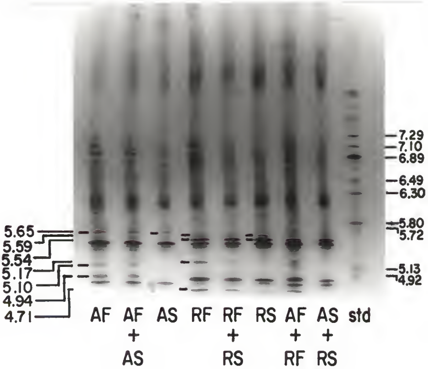


Figure 42. IEF patterns of cooked fillet and/or surimi of Alaska pollock and red hake extracted with urea (cathode on top). Sample mixtures were prepared as shown by the abbreviations. The numerical designations indicate apparent pI values of the protein bands. AF: Alaska pollock fillet, AS: Alaska pollock surimi, RF: red hake fillet, and RS: red hake surimi.

4.71 bands of red hake were missing in their respective surimi samples and therefore could be used for identification purposes.

Conclusion

A good method for the identification of the species origin of surimi products has never been reported in the literature. The SDS-PAGE and IEF methods were shown in this study to be effective in distinguishing the species specificity between Alaska pollock and red hake of raw or cooked fish and surimi samples. Although water is a good protein extractant for raw samples, it does not provide the specificity needed for cooked samples. The use of SDS or urea, however, will increase the number of proteins extracted from cooked samples of fish or surimi and thus improve the effectiveness of species differentiation with SDS-PAGE or IEF. This method appears to have potential application for identifying raw and cooked fish species and surimi samples.

REFERENCES

Alt, F.W., Blackwell, K.B., and Yancopoylos, G.D. 1987. Development of the primary antibody repertoire. Science 238: 1079.

An, H., Marshall, M.R., Otwell, W.S., and Wei, C.I. 1988. Electrophoretic identification of raw and boiled shrimp using various protein extraction systems. J. Food Sci. 53: 313.

An, H., Marshall, M.R., Otwell, W.S., and Wei, C.I. 1989a. Species identification of raw and cooked shrimp by a urea gel isoelectric focusing technique. J. Food Sci. 54: 233.

An, H., Wei, C.I., J. Jhao, Marshall, M.R., and Lee, C.M. 1989b. Electrophoretic identification of fish species used in surimi products. J. Food Sci. 54: 253.

Anonymous. 1986. Hoefer Scientific Instruments. p. 124.

Anonymous. 1988. Pharmacia 52-1464-00-06.

AOAC. 1984. "Official Method of Analysis" 14th ed. Association of Official Analytical Chemists, Washington, D.C.

Babbit, J.K. 1986. Suitability of seafood species as raw materials. Food Technol. 40(3): 97.

Barratt, D.H.P. 1980. Cultivar identification of Vicia fava (L.) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of seed globulins. J. Sci. Food Agric. 31: 813.

Benjamin, D.C., Berzofsky, J.A., East, I.J., Gurd, F.R.N., Hannum, C., Leach, S.J., Margoliash, E., Michael, J.G., Miller, A., Prager, E.M., Reichlin, M., Sercarz, E.E., Smith-Gill, S.J., Todd, P.E., and Wilson, A.C. 1984. The antigenic structure of proteins: A reappraisal. Ann. Rev. Immunol. 2: 67.

Boulikas, T. 1985. Electrophoretic separation of histones and high-mobility-group proteins on acid-urea-triton gels. Anal. Biochem. 149: 379.

Brandon, D.L., Bates, A.H., and Friedman, M. 1988. Enzyme-linked immunoassay of soybean Kunitz trypsin inhibitor using monoclonal antibodies. J. Food Sci. 53: 102.

Brandon, D.L., Haque, S., and Friedman, M. 1987. Interaction of monoclonal antibodies with soy bean trypsin inhibitors. J. Agric. Food Chem. 35: 195.

Burnette, W.N. 1981. "Western blotting": Electrophoretic transfer of proteins form sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. 112: 195.

Caldironi, H.A. and Bazan, N.G. 1980. Quantitative determination of low-salt soluble protein patterns of bovine muscles cooked at different temperatures, by sodium dodecyl sulfate-polyacrlyamide gel electrophoresis. J. Food Sci. 45: 901.

Cantor, C.R. and Schimmel, P.R. 1980. "Biophysical Chemistry" Part. 1., W.H. Freeman and Co., San Francisco, CA.

Catsimpoolas, N. 1975. Isoelectric focusing: Fundamental aspects. Separ. Sci. 10: 55.

Catsimpoolas, N. 1977. Protein separation and analysis by electrophoretic methods: An overview of principles. Ch. 5. In "Food Proteins," J.R. Whitaker and S.R. Tannenbaum (Ed.), Avi Pub. Co., Westport, CT.

Chang, P.R. and Sosulsky, F.W. 1985. Functional properties of dry milled fractions from wild oats (Avena fatua L.). J. Food Sci. 50: 1143.

Chiles, T.C., O'Brien, T.W., and Kilberg, M.S. 1987. Production of mono-specific antibodies to a low abundance hepatic membrane protein using nitrocellulose immobilized protein as antigen. Anal. Biochem. 163: 136.

Chrambach, A. and Baumann, G. 1976. Isoelectric focusing on polyacrylamide gel. Ch. 4. In "Isoelectric Focusing," N. Catsimpoolas (Ed.), Academic Press, New York, NY.

Clark, B.R. and Engvall, E. 1980. Enzyme-linked immunosorbent assay (ELISA): Theoretical and practical aspects. Ch. 8. In "Enzyme-Immunoassay," E.T. Maggio (Ed.), CRC press, Inc., Boca Raton, FL.

Cooper, T.G. 1977. "The Tools of Biochemistry," John Wiley & Sons, New York, NY.

Davis, C.E. and Anderson, J.B. 1984. Size exclusion/HPLC of heated water soluble bovine and porcine muscle proteins. J. Food Sci. 49: 598.

Dimitriadis, G.J. 1979. Effect of detergents on antibody-antigen interaction. Anal. Biochem. 98: 445.

Douillard, J.Y. and Hoffman, T. 1983. Enzyme-linked immunosorbent assay for screening monoclonal antibody production using enzyme-labeled second antibody. Meth. Enzymol. 92: 168.

Dowdie, O.G. and Biede S.L. 1983. Influence of processing temperature on the distribution of tissue and water-soluble proteins in blue crabs (*Callinectes Sapidus*). J. Food Sci. 48: 804.

Dunbar, B.S. 1987. "Two-Dimensional Electrophoresis and Immunological Techniques," Plenum Press, New York, NY.

Duncan, R. and Hershey, J.W.B. 1984. Evaluation of isoelectric focusing running conditions during two-dimensional isoelectric focusing/sodium dodecyl sulfate- polyacrylamide gel electrophoresis: Variation of gel patterns with changing conditions and optimized isoelectric focusing conditions. Anal. Biochem. 138: 144.

Dunker, A.K. and Rueckert, R.R. 1969. Observations on molecular weight determinations on polyacrylamide gel. J. Biol. Chem. 244: 5074.

Edwards, P.A.W. 1981. Some properties and applications of monoclonal antibodies. Biochem. J. 200: 1.

Engvall, E. 1980. Enzyme immunoassay ELISA and EMIT. Meth. Enzymol. 70: 419.

Erickson, M.C., Gordon, D.T., and Anglemier, A.F. 1983. Proteolytic activity in the sarcoplasmic fluids of parasitized pacific whiting (*Merluccius productus*) and unparasitized true cod (*Gadus macrocephalus*). J. Food Sci. 48: 1315.

Ey, P.L., Prowse., S.J., and Jenkin, C.R. 1978. Isolation of pure IgG_1, IgG_{2a}, and IgG_{2b} immunoglobulins from mouse serum using protein A-Sepharose. Immunochem. 15: 429.

Foegeding, A. 1988. Thermally induced changes in muscle proteins. Food Technol. 42: 58.

Foster, C.S. 1982. Lymphocyte hybridomas. Cancer Treat. Rev. 9: 59.

Fullington, J.G., Cole, E.W., and Kasarda, D.D. 1980. Quantitative SDS-PAGE of total protein from different wheat varieties. J. Sci. Food Agric. 31: 43.

Galfre, G. and Milstein, C. 1983. Preparation of monoclonal antibodies: strategies and procedures. Meth. Enzymol. 73: 3.

Gelsema, W.J., De Ligny, C.L., and Van der Veen, N.G. 1979. Isoelectric points of proteins determined by isoelectric focusing in the presence of urea and ethanol. J. Chromatogra. 171: 171.

Gershoni, J.M. and Palade, G.E. 1983. Protein blotting: Principles and applications. Anal. Biochem. 131: 1.

Goding, J.W. 1982. Antibody production by hybridomas. J. Immunol. Methods 39: 285.

Goding, J.W. 1983. "Monoclonal Antibodies: Principles and Practice," Academic Press, Inc., New York, NY.

Goll, D.E., Robson, R.M., and Stromer, M.H. 1977. Muscle proteins. Ch. 6. In "Food Proteins," J.R. Whitaker and S.R. Tannenbaum (Ed.), p. 121. Avi Pub. Co., Westport, CT.

Hamilton, W.D. 1982. Fish species identification by thin layer agarose isoelectric focusing and densitometric scanning. J. Assoc. Off. Anal. Chem. 65: 119.

Hamilton, R.J. and Sewell, P.A. (Ed.) 1982. Introduction to high performance liquid chromatography. Chapman and Hall. New York, NY.

Harris, H. and Hopkinson, D.A. (Ed.) 1976. Principles of enzyme staining. Ch. 2. In "Handbook of Enzyme Electrophoresis in Human Genetics," North-Holl and Pub. Co., N.Y.

Hayden, A.R. 1977. Detection of chicken flesh in beef sausages. J. Food Sci. 42: 1189.

Hayden, A.R. 1978. Determination of residual species serum albumin in adulterated ground beef. J. Food Sci. 43: 476.

Hayden, A.R. 1981. Use of antisera to heat-stable antigens of adrenals for species identification in thoroughly cooked beef sausages. J. Food Sci. 46: 1810.

Hitchcock, C.H.S., Bailey, F.J., Crimes, A.A., Dean, D.A.G., and Davis, P.J. 1981. Determination of soya proteins in food using an enzyme-linked immunosorbent assay procedure. J. Sci. Food Agric. 32: 157.

Hood, L.E., Weissman, I.L., and Wood, W.B. 1984. "Immunology," Benjamin/Cummings Pub. Co., Menlo Park, CA.

Johnson, E. and Stevenson, R. 1978. "Basic Liquid Chromatography," Varian Associates, Inc., Palo Alto, CA.

Kang'ethe, E.K., Jones, S.J., and Patterson, R.L.S. 1982. Identification of the species origin of fresh meat using an enzyme-linked immunosorbent assay procedure. Meat Sci. 7: 229.

Kao, K.J. and Klein, P.A. 1986. A monoclonal antibody-based enzyme-linked immunosorbent assay for quantitation of plasma thrombospondin. Am. J. Clin. Pathol. 86: 317.

Karpas, A.B., Myers, W.L., and Serge, D. 1970. Serological identification of species of origin of sausage meats. J. Food Sci. 35: 150.

Keung, W.M., Ditlow, C.C., and Vallee, B.L. 1985. Identification of human alcohol dehydrogenases isozymes by disc polyacrylamide gel electrophoresis in 7 M urea. Anal. Biochem. 151: 92.

King, N.L. 1984. Species identification of cooked meats by enzyme-staining of isoelectric focusing gels. Meat Sci. 11: 59.

King N.L. and Kurth, L. 1982. Analysis of raw beef samples for adulterant meat species by enzyme-staining of isoelectric focusing gels. J. Food Sci. 47: 1608.

Kinsella, J.E. 1988. Fish and seafoods: Nutritional implications and quality issues. Food Technol. 42: 146.

Knudsen, K.A. 1985. Proteins transferred to nitrocellulose for use as immunogens. Anal. Biochem. 147: 285.

Kohler, G. and Milstein, C. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256: 495.

Koohmaraie, M., Kennick, W.H., Anglemier, A.F., Elgasim, E.A., and Jones, T.K. 1984. Effect of postmortem storage on cold-shortened bovine muscle: Analysis by SDS-polyacrylamide gel electrophoresis. J. Food Sci. 49: 290.

Krzynowek, J. and Wiggin, K. 1979. Identification of species in cooked crab meat by thin layer isoelectric focusing. J. Assoc. Off. Anal. Chem. 62: 630.

Krzynowek, J. and Wiggin, K. 1981. Generic identification of cooked and frozen crab meat by thin layer polyacrylamide gel isoelectric focusing: Collaborative study. J. Assoc. Off. Anal. Chem. 64: 670

Kurth, L. and Shaw, F.D. 1983. Identification of the species of origin of meat by electrophoretic and immunological methods. Food Technol. Australia 35: 328.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680.

Lanier, T.C. 1986. Functional properties of surimi. J. Food Sci. 40: 107.

Lazure, C., Dennis, M., Rochemont, J., Seidah, N.G., and Chretien, M. 1982. Purification of radiolabeled and native polypeptides by gel permeation high-performance liquid chromatography. In "High-Performance Liquid Chromatography of Proteins and Peptides," M.T.W. Hearn, F.E. Regnier, and C.T. Wehr (Ed.), p. 141. Academic Press, New York, NY.

Lee, C.M. 1984. Surimi process technology. Food Technol. 38(11): 69.

Lee, C.M. 1986a. Surimi manufacturing and fabrication of surimi-based products. Food Technol. 40(3): 115.

Lee, C.M. 1986b. A pilot plant study of surimi making properties of red hake (Urophycis chuss). Ch. 5. In "International Symposium on Engineered Seafood Including Surimi," R. Martin and R. Collette (Ed.), p. 225, National Fisheries Institute, Washington, D.C.

Lee, Y.B., Rickansrud, D.A., Hagberg, E.C., and Brisky, E.J. 1974. Application of SDS-acrylamide gel electrophoresis for determination of the maximum temperature to which bovine muscle have been cooked. J. Food Sci. 39: 428.

Lin, T.S. and Lanier, T.C. 1980. Determination of species composition in minced fish blocks. Presented at "Third National Technical Seminar on Mechanical Recovery and Utilization of Fish Flesh," Raleigh, NC, Dec. 1-3.

Lin, T.S., Su, H.K., and Lanier, T.C. 1980. Characterization of fish muscle proteases using radio-labeled protein substrates. J. Food Sci. 45: 1036.

Littlefield, J.W. 1964. Selection of hybrids from matings of fibroblasts in vitro and their presumed recombinants. Science 145: 709.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 256.

Lundstrom, R.C. 1980. Fish species identification by thin layer polyacrylamide gel isoelectric focusing: Collaborative study. J. Assoc. Off. Anal. Chem. 63: 69.

Lundstrom, R.C. 1981a. Fish species identification by isoelectric focusing: Sarcoplasmic protein polymorphism in Monkfish (Lophius americanus). J. Assoc. Off. Anal. Chem. 64: 32.

Lundstrom, R.C. 1981b. Rapid fish species identification by agarose isoelectric focusing of sarcoplasmic proteins. J. Assoc. Off. Anal. Chem. 64: 38.

Lundstrom, R.C. 1983a. Fish species identification by agarose gel isoelectric focusing: Collaborative study. J. Assoc. Off. Anal. Chem. 66: 123.

Lundstrom, R.C. 1983b. Identification of Pacific rockfish (Sebastes) species by isoelectric focusing. J. Assoc. Off. Anal. Chem. 66: 974.

Lundstrom, R.C. 1984. Identification of species in cooked seafood using monoclonal antibodies. In Proceedings of the "Symposium on Meat and Fish Identification," held at the 9th Annual A.O.A.C. Spring Workshop, Assn. Foods and Drug Officials, Philadelphia, PA.

Lundstrom, R.C. 1985. The potential use of monoclonal antibodies for identification of fish stocks. p. 137. Presented at the "Stock Identification Workshop: Recent Innovations and State of the Art," National Marine Fisheries Service, Panama City, FL, Nov. 5-7.

Marshall, T. and Williams, K.M. 1988. High resolution two-dimensional electrophoresis and sodium dodecyl sulphate-polyacrylamide gel electrophoresis of cheese proteins after rapid solubilization. Electrophoresis 9: 143.

Martin R.E., Doyle, R.H., and Brooker, J.R. 1983. Toward an improved seafood nomenclature system. Marine Fish. Rev. 45(7-8-9): 1.

McCausland, J. and Wrigley, C. W. 1976. Electrophoretic analysis of wheat and rye mixtures in meal, flour and baked goods. J. Sci. Food Agric. 27: 1197.

McCormick, R.J., Kropf, D.H., Reeck, G.R., Hunt, M.C., and Kastner, C.L. 1987. Effect of heating temperature and muscle type on porcine muscle extracts as determined by reverse phase high performance liquid chromatography. J. Food Sci. 52: 1481.

Miller, D.W. and Elgin, S.C.R. 1974. Isoelectric focusing of proteins exposed to sodium dodecyl sulfate. Anal. Biochem. 60: 142.

Neal, J.V., Rosenblum, B.B., Sing, C.F., Skolnick, M.M., Hanash, S.M., and Sternberg, S. 1984. Adapting two-dimensional gel electrophoresis to the study of human germ-line mutation rates. Ch. 9. In "Two-Dimensional Gel Electrophoresis of Proteins," J.E. Celis and R. Bravo (Ed.), Academic Press, Inc., New York, NY.

Oellingrath, I.M. 1988. Heat degradation of heme in methemoglobin and metmyoglobin model systems measured by reversed-phase ion-pair high performance liquid chromatography. J. Food Sci. 53: 40.

O'Farrell, P.H. 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. 250: 4007.

Osman, M.A., Ashoor, S.H., and Marsh, P.C. 1987. Liquid chromatographic identification of common fish species. J. Assoc. Off. Anal. Chem. 70: 618.

Patterson, R.M., Whittaker, R.G., and Spencer, T.L. 1984. Improved species identification of raw meat by double sandwich enzyme-linked immunosorbent assay. J. Sci. Food Agric. 35: 1018.

Pesce, A.J., Ford, D.J., Gaizutis, M., and Pollak, V.E. 1977. Binding of protein to polystyrene in solid-phase immunoassays. Biochim. Biophys. Acta 492: 399.

Pesce, A.J., Ford, D.J., and Makler, M.T. 1981. Properties of enzyme immunoassays. Ch. 3. In "Enzyme Immunoassay," E. Ishikawa, T. Kawai and K. Miyai (Ed.), Igaku-shoin Med. Pub. Inc., New York, NY.

Pryde, A. and Gilbert, M.T. 1979. "Applications of High Performance Liquid Chromatography," Chapman and Hall, New York, NY.

Rabilloud, T., Vincens, P., and Tarroux, P. 1985. A new tool to study genetic expression using 2-D electrophoresis data; the functional map concept. Fed. Eur. Biochem. Soc. 189: 171.

Rasekh, J.G., Waters, M.E., and Sidwell, V.D. 1980. The effect of washing on the quality characteristics of minced fresh Croaker, Micropogon undulatus, held in frozen storage. Marine Fish. Rev. 42(11): 26.

Regenstein, J. and Lanier, T.C. 1986. Surimi: Boon or Boondoggle? Seafood Leader 6: 152.

Regenstein, J.M. and Stamn, J.R. 1979. Factors affecting the sodium chloride extractability of muscle proteins from chicken breast, trout white and lobster tail muscle. J. Food Biochem. 3: 191.

Regnier, F.E. 1982. High-performance ion-exchange chromatography of proteins: The current status. In "High-Performance Liquid Chromatography of Proteins and Peptides," M.T.W. Hearn, F.E. Regnier, and C.T. Wehr (Ed.), p. 1. Academic Press, New York, NY.

Reynolds, J.A. and Tanford, C. 1970. The gross conformation of protein-sodium dodecyl sulfate complexes. J. Biol. Chem. 245: 5161.

Righetti, P.G. and Drysdale, J.W. 1974. Isoelectric focusing in gels. J. Chromatography 98: 271.

Rilbe, H. 1976. Theoretical aspects of steady-state isoelectric focusing. In "Isoelectric Focusing," N. Catsimpoolas (Ed.), p. 13. Academic Press, New York, NY.

Scope, R.K. 1970. Characterization and study of sarcoplasmic proteins. In "The Physiology and Biochemistry of Muscle as a Food," 2nd ed., E.J. Briskey, R.G. Cassens, and B.B. Marsh (Ed.), p. 471. The University of Wisconsin Press, Madison, WI.

Sewell, P.A. and Clarke, B. 1987. "Chromatographic Separations," John Wiley and Sons, New York, NY.

Shuster, L. 1971. Preparative acrylamide gel electrophoresis: Continuous and disc techniques. Meth. Enzymol. 22: 412.

Sinkovics, J.G. and Dreesman, G.R. 1983. Monoclonal antibodies of hybridomas. Rev. Infect. Dis. 5: 9.

Skerritt, J.H. 1985. A sensitive monoclonal-antibody-based test for gluten detection: Quantitative immunoassay. J. Sci. Food Agric. 36: 987.

Skerritt, J.H., Diment, J.A., and Wrigley, C.W. 1985. A sensitive monoclonal-antibody-based test for gluten detection: Choice of primary and secondary antibodies. J. Sci. Food Agric. 36: 995.

Skerritt, J.H. and Smith, R.A. 1985. A sensitive monoclonal-antibody-based test for gluten detection: Studies with cooked or processed foods. J. Sci. Food Agric. 36: 980.

Stevenson, J.R. 1985. Dynamics of the integument. Ch.1. In "The Biology of Crustacea," Vol. 9. D.E. Bliss and L.H. Mantel (Ed.), Academic Press, Inc. New York, NY.

Stumph, W.E., Elgin, S.R., and Hood, L. 1974. Antibodies to protein dissolved in sodium dodecyl sulfate. J. Immunol. 113: 1752.

Su, H., Lin., T.S., and Lanier, T.C. 1981. Investigation into potential sources of heat-stable alkaline protease in mechanically separated Atlantic croaker (Micropogon undulatus). J Food Sci. 46: 1654.

Sutton, J.G., Goodwin, J., Horscroft, G., Stockdale, R.E., and Frake, A. 1983. Identification of trout and salmon bloods by simple immunological technique and by electrofocusing patterns of red cell enzyme superoxide dismutase. J. Assoc. Off. Anal. Chem. 66: 1164.

Svensson, H. 1961. Isoelectric fractionation, analysis, and characterization of ampholytes in natural pH gradients. I. The differential equation of solute concentrations at a steady state and its solution for simple cases. Acta Chem. Scand. 15: 325.

Tanford, C. 1968. Protein denaturation. Adv. Prot. Chem. 23: 121.

Thakker, K.D. and Grady, L.T. 1984. Solubility. Ch. 2. In "Food Analysis," D.W. Gruenwedel and J.R. Whitaker (Ed.), Marcel Dekker, Inc., New York, NY.

Tjian, R., Stinchcomb, D., and Losick, R. 1974. Antibody directed against Bacillus subtilis sigma factor purified by sodium dodecyl sulfate slab gel electrophoresis. J. Biol. Chem. 250: 8824.

Toom, P.M., Ward, C.F., and Weber, J.R. 1983. Identification of species by isoelectric focusing. In "Chemistry and Biochemistry of Marine Food Products," R.E. Martin, G.J. Flick, C.E. Heberd, and D.R. Ward (Ed.), Avi Pub. Co. Westport, CT.

Tonegawa, S. 1983. Somatic generation of antibody diversity. Nature 302: 575.

Towbin, H. and Gordon, J. 1984. Immunodotblotting and dot immunobinding-Current status and outlook. J. Immunol. Meth. 72: 313.

Tracy, R.P., Katzman, J.A., Kimlinger, T.K., Hurst, G.A., and Young, D.S. 1983. Development of monoclonal antibodies to proteins separated by two-dimensional gel electrophoresis. J. Immunol. Meth. 65: 97.

Tuszynski, G.P., Baker, S.R., Fuhrer. J.P., Buck, C.A., and Warren, L. 1978. Glycopeptides derived from individual membrane glycoproteins from control and Rous sarcoma virus-transformed hamster fibroblasts. J. Biol. Chem. 253: 6092.

Van Oss, C.J., Good, R.J., and Chaudhury, M.K. 1987. Mechanism of DNA (Southern) and protein (Western) blotting on cellulose nitrate and other membranes. J. Chromatography 391: 53.

Vesterberg, O. 1969. Synthesis and isoelectric fractionation of carrier ampholytes. Acta Chem. Scand. 23: 2653.

Vesterberg, O. 1971. Isoelectric focusing of proteins. Meth. Enzymol. 22: 389.

Vesterberg, O. 1976. The carrier ampholytes. In "Isoelectric Focusing," N Catsimpoolas (Ed.), p. 53. Academic Press, New York, New York.

Vondruska, 1988. Seafood consumption, availability, and quality. Food Technol. 42: 168.

Wallis, C., Melnick, J.L., and Gerba, C.P. 1979. Concentration of viruses from water by membrane chromatography. Ann. Rev. Microbiol. 33: 413.

Weber, K. and Kuter, D.J. 1971. Reversible denaturation of enzymes by sodium dodecyl sulfate. J. Biol. Chem. 246: 4504.

Weber, K. and Osborn, M. 1969. The reliability of molecular determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. 244: 4406.

Weber, K. and Osborn, M. 1975. Proteins and sodium dodecyl sulfate: Molecular weight determination on polyacrylamide gels and related procedures. Ch. 3. In "The Proteins," 3rd ed. H. Neurath and R.L. Hill (Ed.), p 179. Academic Press, New York, NY.

Weber, K., Pringle, J.R., and Osborn, M. 1972. Measurement of molecular weights by electrophoresis on SDS-acrylamide gel. Meth. Enzymol. 26: 3.

Westermeier, R., Potel, W., Gorg, A., and Telek, L. 1985. Isoelectric focusing and sodium dodecylsulphate electrophoresis of buffer soluble seed proteins of twelve varieties of winged bean (Psophocarpus tetragonolobus L.). J. Sci. Food Agric. 36: 135.

Whittaker, R.G., Spencer, T.L., and Copland, J.W. 1983. An enzyme-linked immonosorbent assay for species identification of raw meat. J. Sci. Food Agric. 34: 1143.

Williams, A.B. 1984. "Shrimps, Lobsters, and Crabs of the Atlantic Coast of the Eastern United States, Maine to Florida," Smithsonian Inst. Press, Washington, DC.

Yamada, J. and Suzuki, A. 1982. Identification of fish species by thin layer isoelectric focusing of sarcoplasmic protein. Bull. Jap. Soc. Sci. Fish. 48: 73.

Yowell, K. and Flurkey, W. 1986. Effect of freezing and microwave heating on proteins from codfish fillets: Analysis by SDS polyacrylamide gel electrophoresis. J. Food Sci. 51: 508.

Zola, H. and Brooks, D. 1982. Techniques for the production and characterization of monoclonal hybridoma antibodies. Ch. 1. In "Monoclonal Hybridoma Antibodies: Techniques and Applications," J.G.R. Hurrell (Ed.), CRC Press, Inc. Boca Raton, FL.

BIOGRAPHICAL SKETCH

Haejung An was born on May 13, 1958 in Seoul, Korea. After she graduated from the Soong-eui Girls' High School in Seoul in January, 1977, she entered the Seoul National University in March of the same year and received a Bachelor of Science degree in February, 1981. She joined Louisiana State University at Baton Rouge in January, 1982, and received her Master of Science degree in food science. She continued her graduate studies in the Department of Food Science and Human Nutrition at the University of Florida, Gainesville, and is expected to receive the degree of Doctor of Philosophy in May, 1989.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Cheng-i Wei

Cheng-i Wei, Chairman
Professor of Food Science
and Human Nutrition

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

M R Marshall

Maurice R. Marshall,
Co-chairman
Associate Professor of Food
Science and Human Nutrition

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

W.S. Otwell

W. Steven Otwell
Professor of Food Science
and Human Nutrition

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Paul A. Klein

Paul A. Klein
Professor of Pathology
and Lab. Medicine

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

K. J. Kao
Assistant Professor of
Pathology and Lab. Medicine

This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

May, 1989

Jack L. Fry
Dean, College of Agriculture

Dean, Graduate School